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# Long Live The Queen: influence of membrane phospholipids on longevity of honey bees

Nicolas Martin
*University of Wollongong*

UNIVERSITY OF WOLLONGONG AUSTRALIA

# Long Live The Queen: influence of membrane phospholipids on longevity of honey bees

Nicolas Martin, *BSc* (Hon1), *MSc*,

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Doctor of Philosophy

Supervisors:
Professor Paul L. Else
Emeritus Professor A. J. Hulbert
Associate Professor Todd W. Mitchell
The University of Wollongong School of Medicine

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# Declaration

I, *Nicolas Martin*, declare that this thesis is submitted in partial fulfilment of the requirements for the conferral of the degree *Doctor of Philosophy*, from the University of Wollongong, is wholly my own work unless otherwise referenced or acknowledged. This document has not been submitted for qualifications at any other academic institution.

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All experiments were conceptualized by *Nicolas Martin*, *A. J. Hulbert*, *Todd W. Mitchell* and *Paul L. Else*. *Nicolas Martin* acquired all the data, did all the formal analysis and wrote the first draft of all the manuscripts. *A. J. Hulbert*, *Todd W. Mitchell* and *Paul L. Else* supervised, reviewed and edited the manuscripts.

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**Nicolas Martin**

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# Dedication

This thesis is dedicated to my parents, *Michel* and *Hélène* who always supported me throughout my whole life, and my nan, *Rita*, who has been an inspiration along the journey of my *Ph.D.* As long as I can remember, nan always wanted to have a better education than the one she had, leaving School in Year 7 after a shocking car accident. She is very grateful that I studied honey bees for my *Ph.D.* and always dreamed about coming to Australia. Thanks for your endless support nan!

# Abstract

This thesis aimed to use a social insect model to investigate the effect of membrane phospholipid composition on longevity. The two female castes of honeybees are genetically identical yet become either exceptionally long-lived queens (up to 8 years) or short-lived workers (typically 2-6 weeks). This distinction is based on dietary supply during the larval developmental stage where queens are immersed in liquid food (royal jelly) until metamorphosis (pupation) whereas workers get a restricted supply of royal jelly. After emergence, workers begin feeding on honey and pollen ('bee bread') with pollen containing a high level of polyunsaturated fatty acids (PUFA), whereas queens feed exclusively on royal jelly with negligible PUFA (assumed to be the same jelly they receive as larvae). PUFA are capable of significant levels of oxidation (i.e. peroxidation) whereas monounsaturated (MUFA) and saturated (SAT) fatty acids are very resistant to peroxidation. Therefore, this thesis investigated the role of dietary lipids (specifically PUFA) and its potential to determine longevity differences in female honeybees.

Membrane phospholipids of six different life-history stages, covering all developmental stages from little larva to old adults of the castes of the honey bees (*Apis mellifera*) were analysed using shotgun mass spectrometry lipidomics. The membrane phospholipids of workers and queens as larvae, pupae, and newly emergent adults all had low PUFA and high MUFA levels. However, queens continued to maintain low PUFA and high MUFA levels throughout their entire lives whereas, by day 4 of adult life, worker bees had increased their PUFA levels 5-fold, with a commensurate decrease in MUFA. This difference in membrane composition appears due to the difference in nutrition after adult emergence. The increase in PUFA in worker membranes occurred in all phospholipid classes but predominantly in phosphatidylcholine (PC) and phosphatidylethanolamine (PE) molecules. The low level of PUFA present in the membranes of queens was mostly found in phosphatidylinositol (PIn) molecules. Queens maintained a much higher abundance of phospholipid molecules containing SFA and MUFA (that are both resistant to peroxidation and protective of phospholipids that contain PUFA) compared to adult worker bees. Both female castes shared a similar level of total phospholipids. Adult queens also possessed a much higher abundance of their PUFA-containing phospholipids as plasmalogens (that limit lipid peroxidation) compared to worker bees. Overall, queen membranes appear to be far

more resistant to peroxidation compared to worker membranes.

The remodeling of membrane phospholipids in worker bees was found to be relatively fast, occurring primarily over the first four days of adult life. This remodeling could be divided into three distinct steps. Firstly, an increase in total membrane phospholipid in the first 24 hours post-emergence. The increased in total phospholipid is best explained by a heightened *de novo* synthesis of phospholipid molecules containing SFA and MUFA. Secondly, a progressive decrease in the concentration of newly synthesized phospholipids, and their replacement by PUFA-containing phospholipid molecules (peaking by day 4). The final net result was the production of PUFA-containing phospholipids at the expense of newly synthesized SFA and MUFA-containing phospholipids, culminating in elevated stable levels of total phospholipids in worker bees 48 hours post-emergence. This remodeling of worker bee membranes suggests that PUFA acquired from the diet is being incorporated into pre-existing, newly formed phospholipid molecules containing SFA and MUFA.

Membrane composition has been related to maximum lifespan in mammals, birds, bivalve molluscs, and the nematode *Caenorhabditis elegans*. In all cases, a long maximum lifespan is associated with low PUFA content in membrane lipids. The results from the membrane phospholipids characterised at the different life-history stages showed that female honeybees follow the same relationship. An experimental test of the hypothesis, that differences in membrane lipid composition are associated with maximum lifespan, was conducted using worker bees. Based on the findings that long-lived queen bees have membranes with a low level of PUFA content, it was postulated that a diet deprived of PUFA would maintain a low level of PUFA in worker membranes and increase their lifespan. To test this hypothesis, four large populations ($n > 230$ for each population) of newly-emergent adult workers were fed isoenergetic diets that differed in their fatty acid composition; two with PUFA (honey+pollen; honey+casein+PUFA), and two without PUFA (honey+yeast; honey+casein). Emergent adult worker bees were housed in an experimental hive maintained in an indoor room at 31°C and 60% relative humidity to create free-living hive conditions. The diets containing PUFA produced membrane PUFA levels that were similar to those of normal free-living worker bees, while worker bees on the PUFA-deficient diets developed membranes with a low level of PUFA (i.e. similar to adult queens). The results showed that dietary lipids did not affect the average lifespan of the different dietary groups. However, the

maximum lifespan (average longevity of longest-living 10% of the population) of worker bees on the PUFA-deficient diets increased by up to 30% compared to those on the PUFA-containing diets.

Lipofuscin fluorescent pigments (AGE) have been proposed as a good biomarker for cellular senescence. Lipofuscin pigments were measured by spectrofluorometry in several life-history stages of bees covering development and adult life of workers and queens. Measurement of lipofuscin fluorescent pigments showed a similar trend during development for both female castes. Both castes initially increased their level of lipofuscins, but queens had a higher level than workers when emerging as adults. Lipofuscin levels decreased in both castes during adult life, suggesting that both workers and queens possess a mechanism(s) to dispose of lipofuscins with ageing. The two castes had similar levels of lipofuscins during adult life, suggesting that lipofuscins are unlikely to explain the large difference in adult lifespan. Likewise, workers and age-matched queens had similar metabolic rates, suggesting that differences in metabolism are also unlikely to explain differences in lifespan.

Few studies have previously used the large difference in lifespan between queens and workers to examine the mystery of ageing. Instead, most previous studies have characterized changes in worker bees through their life history stages (e.g. transition from nurses to foragers). The results from the current set of experiments are among the first to experimentally test a mechanism that could explain the difference in lifespan. These results suggest that the castes differ significantly in membrane phospholipids during adult life. These differences in membrane phospholipids are explained by a difference in nutrition after emergence. The difference in membrane phospholipids is, however, a partial reason rather than the major reason that explains the tremendous difference in lifespan between the female castes. Social insects offer both challenges and promise to investigate the processes of ageing in a model that has evolved to produce tremendous differences in lifespans between genetically identical sister castes.

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# List of abbreviations

AGE: age-related glycation end-products pigment

AmOAc: ammonium acetate

ANOVA: analysis of variance

BHT: butylated hydroxytoluene

BP: barometric pressure

CAT: catalase

DNA: deoxyribonucleic acid

$CHCl_3$: chloroform

D: drone (male)

E: adult emergent

EDTA: ethylenediaminetetraacetic acid

EGTA: ethylene glycol-bis-β-aminoethyl ether-N,N,N',N'-tetraacetic acid

EL: early larva

FAME: fatty acid methyl ester

GC: gas chromatography

Gpx: glutathione peroxidase

HPLC: high performance liquid chromatography

kPa: kiloPascal

LL: late larva

LPC: lysophosphatidylcholine

LPE: lysophosphatidylethanolamine

MeOH: methanol

mg: milligram

MDA: malondialdehyde

mL: milliliter

mM: millimolar

MS: mass spectrometry

MTBE: tertiary butyl methyl ether

MUFA: monounsaturated fatty acids

NA: not present

nmol: nanomole

NSW: New South Wales, Australia

PBS: phosphate buffered saline

PC: phosphatidylcholine

PE: phosphatidylethanolamine

PI: peroxidation index

PIn: phosphatidylinositol

PS: phosphatidylserine

PUFA: polyunsaturated fatty acids

OA: old adult

PU: pupa

YA: young adult

SFA: saturated fatty acids

SOD: superoxide dismutase

STPD: standard temperature and pressure conditions

STPD: standard temperature and pressure dry conditions

ROS: reactive oxygen species

SEM: standard error of the mean

μg: microgram

*u*L: microliter

*u*M: micromolar

v:v volume: volume ratio

W: workers

w/v: weight: volume ratio

w/w: weight: weight ratio

Q: queens

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# Chapter 1 – Introduction

Ageing is a universal process in animals as life is finite, even for species with negligible senescence. Ageing can be characterised as a process that involves the progressive, endogenous and irreversible deterioration of biochemical processes (Strehler, 1962) that underpin the physiological function and therefore homeostasis of all organisms (Hulbert et al., 2007a). These characteristics suggest that the causes of ageing are present during the whole lifespan of organisms. The rate of ageing affects an individual's lifespan through its influence on the risk of developing life-threatening diseases and deterioration. Despite recent advances in modern medicine and public health, that have contributed significantly to increasing the average lifespan of humans during the last century, the maximum lifespan of humans has remained stubbornly fixed since the early 1900's (Figure 1.1). This suggests that these advances have not altered the ageing process itself. An obvious question when looking at ageing is: What do we do so well when we are young that stops working as we get older?

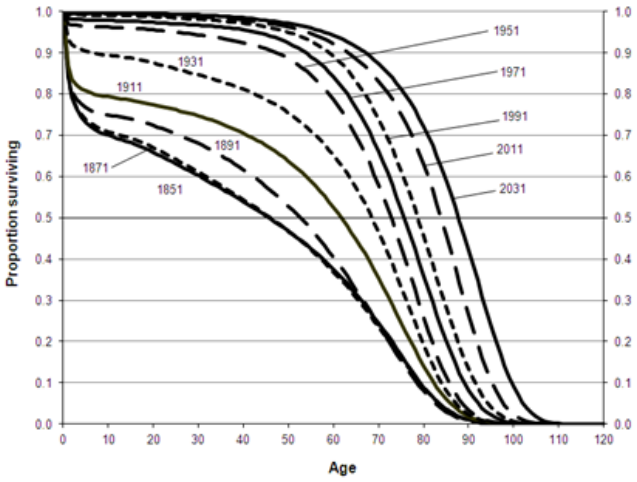


Figure 1. 1. Proportion of survival according to mortality rates experienced or predicted from 1851 to 2031 from the population of Wales, England. From this figure, it can be seen that the median lifespan of the cohort born in 1851 was 46 years, while the predicted value for the cohort born in 2031 is over 90 years. Another interesting observation is a shift toward a more rectangular survival curve with time. The large increase in average lifespan is mainly due to the decrease in infant mortality, the discovery of vaccines and antibiotics, as well as a general improvement in control of infectious diseases and improved nutrition (Viña et al., 2007). Image reproduced from the Office for National Statistics, United Kingdom.

Perhaps one of the first references to ageing goes back to Greek mythology. Eos, the goddess of the Dawn and lover of the Trojan Tithonus, asks Zeus to confer immortality on

Tithonus but forgets to mention eternal youth. Tithonus indeed lived forever, but grew ever-older, babbling endlessly and begging to die. Perhaps the best way to appreciate the curse on Tithonus was captured by Alfred Lord Tennyson (1809-1892) who pictured Tithonus looking on "the dim fields of the homes of happy men that have the power to die, and grassy barrows of the happier death, release me and restore me to the ground." This tale is still relevant in the 21st century as pharmaceutical companies begin to focus their massive resources on the 'fountain of youth,' i.e. the extension of lifespan, despite the fact there is no consensus about what causes ageing, and what determines the rate of ageing. As modern medicine moves forward, potentially extending lifespan, but not necessary 'healthspan', every extra year of life granted by these technologies will likely lead to a few further months spent in good health, and the rest in a terminal decline. Like Tithonus, finally, we may beg for the grave.

## 1.1 What do we know about ageing?

Ageing is associated with a progressive decline in physiological function (Figure 1.2). For example, maximum heart and breathing rates both reduce with age in humans. However, some of the physiological parameters appear to be more affected by age than others. Work rate (measured as $VO_2$) is reduced to 30 % by age 80, while nerve conductivity remains at 90%.

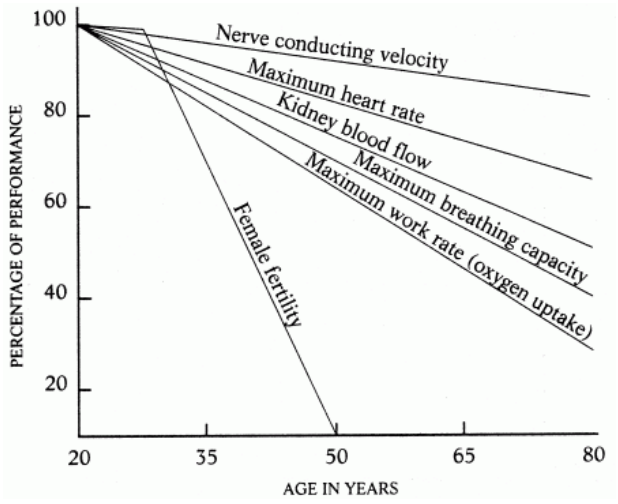


Figure 1. 2. Changes reported in several physiological parameters with ageing in humans. The figure is reproduced from Best, 2010.

Ageing is the biggest risk factor for developing diseases such as Alzheimer's, Parkinson's, dementia, cancer, cardiovascular disease and neurodegeneration (Figure 1.3). In almost all age-related diseases, both the rate and prevalence of diseases are fairly low and constant early on in life but then increases significantly with ageing. For instance, the rate of cancer is very low up to the age of 50 in males and females in the UK. However, after 50, the rate of cancer shows a marked increase, being higher in males compared to females. Understanding ageing is important because 'age' is the dominant risk factor for most disease, and understanding the mechanisms and processes of ageing will likely help increase 'healthspan' in humans.

## 1.2 Hallmarks of ageing

The quest to understand ageing has given rise to many different lines of investigation: including biochemistry, physiology, genetics, and lately, computer and pharmaceutical approaches**.** This research has led to the common features of ageing being separated into several hallmarks (Figure 1.4; López-Otín et al., 2013). Because the hallmarks co-occur during ageing and are interconnected, understanding their causal network may help to discover the importance of the different players in determining the final equation of lifespan. However, the complexity of this equation increases if we consider that each hallmark contains independent factors that can influence its outcome. As an example, epigenetics is influenced by multiple factors such as the state of development, environment, chemicals, temperature, and age, as well as diet (Carey, 2012). Likewise, mitochondrial dysfunction is influenced by levels of free radicals, the fatty acid composition of mitochondrial membranes, the protein composition of the electron transport chain, and oxygen availability. A major challenge in studying ageing is to dissect the interdependence between the candidate hallmarks, and to learn more about their relative contributions to longevity.

## 1.3 Longevity as a means to study ageing

The preferred method used to study ageing is longevity. Organisms achieve different longevities that range from hours to hundreds of years as organisms age at different rates (Table 1.1.). For example, the Greenland shark (*Somniosus microcephalus*) has a maximum lifespan of over 400 years, the bivalve Ocean quahog (*Arctica islandica*) has a maximum

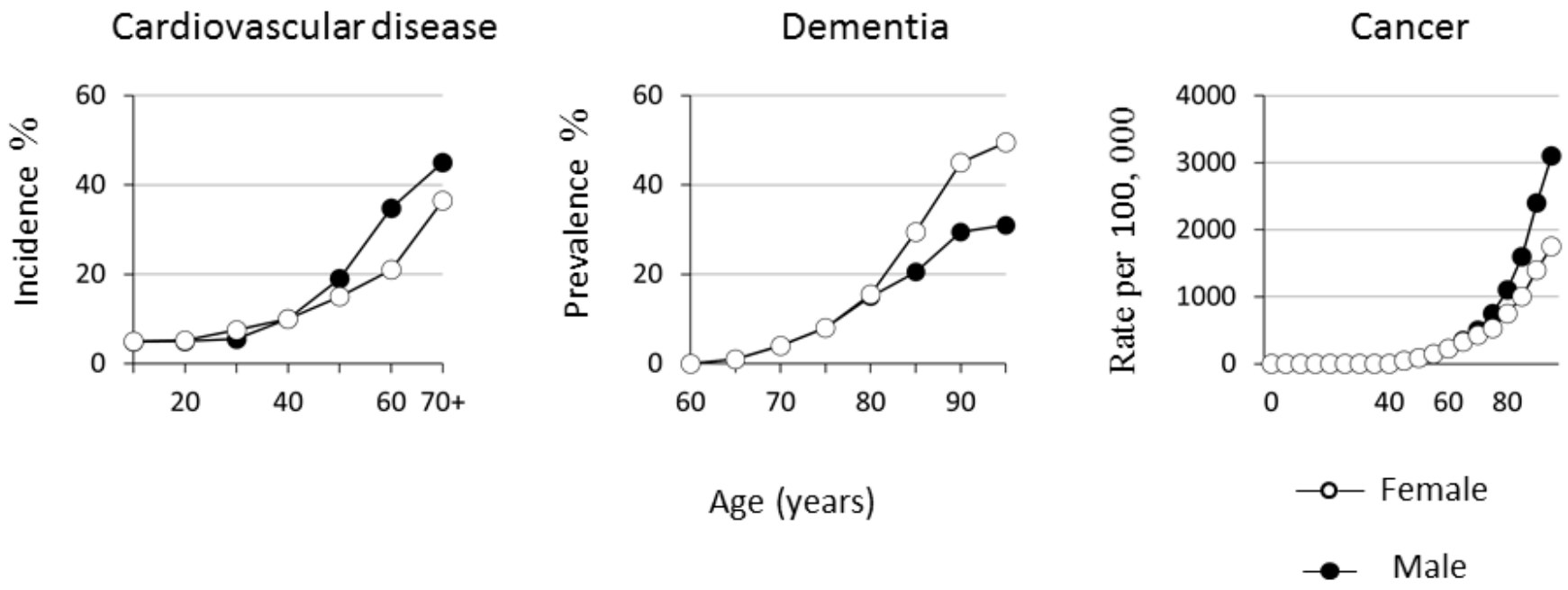


Figure 1. 3. Disease rates for the most common diseases of old age in 2006. Rates of cardiovascular disease incidence in England (Data from British Heart Foundation 'Coronary heart disease statistics'). Rates of dementia prevalence in European countries (data from Alzheimer Europe). Rates of cancer per 100,000 population in the United Kingdom (Data from Cancer Research, United Kingdom).

lifespan of over 507 years, and some other species, such as the so-called immortal jellyfish (*Turritopsis dohrnii*), could have an infinite lifespan. As for humans, Madam Calment is recorded as the longest-living human to date; she lived for 122 years. However, most of the common organism models used in ageing research have a relatively short lifespan (see examples in Table 1.1). One exception is the naked mole rat, with a long lifespan for its body size with a body mass of 28 grams but a maximum lifespan of 31 years compared to a similarly sized mouse with a lifespan of 4 years. The naked mole rat has gained increasing interest as an alternative model for ageing in recent years (Buffenstein, 2005; Rodriguez et al., 2011). Another vertebrate group of interest for living longs are birds. Species of parrots live to nearly 50 years while pigeons which have a similar body mass to rats live for over 18 years. A major challenge in ageing is to distinguish the causes versus the effects of ageing. One way to achieve this goal is to find treatments that can extend the lifespan and, by definition, delay ageing.

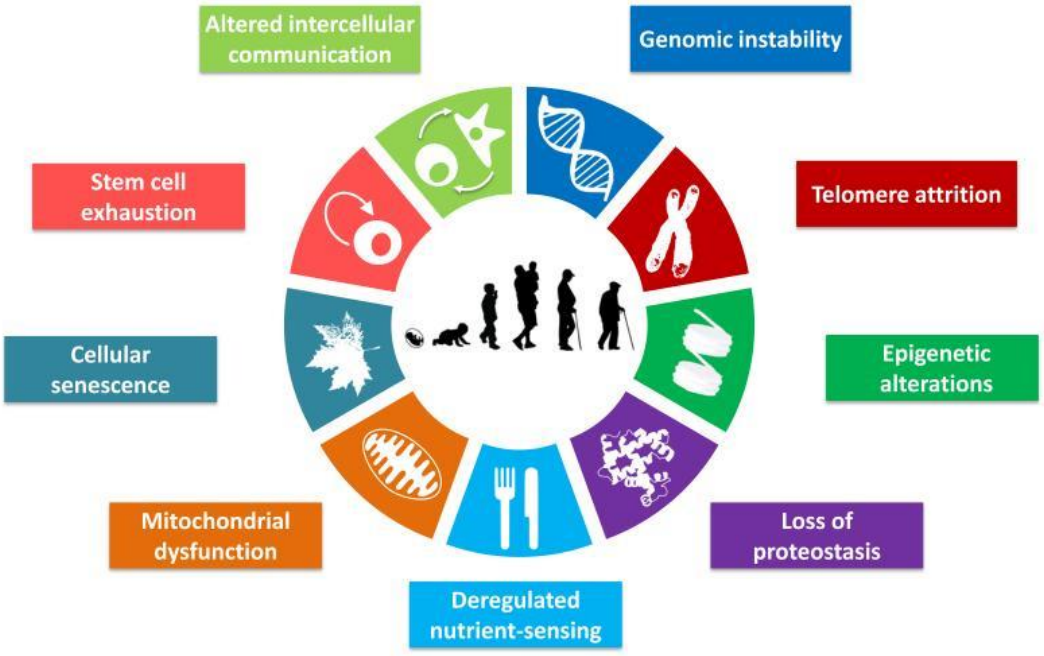


Figure 1. 4. The nine hallmarks of ageing that build up the complex framework of ageing. These hallmarks include genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulation of nutrient-sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication. These hallmarks form the base of the complex framework of ageing. This categorization has helped to conceptualize the essence of ageing and its underlying mechanisms. This figure is reproduced from López-Otin et al., 2013.

Table 1.1. Maximum lifespan of different organisms. Yeast, nematode, fruit fly, mouse and rats are the most common models currently used in ageing research. Data are from An Age Data Base.

| Common name | Scientific name | Maximum lifespan (Years) |
|---|---|---|
| Blue whale | *Balaenoptera musculus* | 110 |
| Human | *Homo sapiens* | 122 |
| Greenland shark | *Somnious microcephalus* | > 400 |
| Ocean quahog | *Arctica islandica* | > 507 |
| Immortal jellyfish | *Turritopsis dohrnii* | ? |
| Yeast | *Saccharomyces cerevisiae* | 0.04 |
| Nematode | *Caenorhabditis elegans* | 0.2 |
| Fruit fly | *Drosophila melanogaster* | 0.3 |
| Mouse | *Mus musculus* | 4 |
| Rat | *Rattus norvegicus* | 4 |
| Naked mole rat | *Heterocephalus glaber* | 31 |
| Common quail | *Coturnix coturnix* | 14.6 |
| Yellow-crested cockatoo | *Cacatua sulphurea* | 49.7 |
| Grey parrot | *Psittacus erithacus* | 49.7 |
| Band-tailed pigeon | *Patagioenas fasciata* | 18.5 |

# 1.4 Treatments extending lifespan and healthspan

Mankind has sought eternal life for hundreds of years and has spectacularly failed to obtain it. One bizarre demonstration was the French physiologist Charles Brown-Séquard who injected himself with extracts from the testicles of dogs and guinea pigs and reported improved vigour and mental powers in 1889. By the end of the year 1889, some 12,000 physicians were administering the Brown-Séquard's Elixir. Surgeons around the world were implanting sliced testicles from various animals such as goats, monkeys, and even prisoners, hoping that humans could obtain a level of immortality from the procedure. The treatment is thought to have had a placebo effect, and Brown-Séquard's image went from that of a brilliant savant to a foolish old man (Aminoff, 2010).

### 1.4.1 Calorie restriction

Before a treatment can be considered to affect ageing, it must extend the lifespan of a wide range of organisms to ensure its universality. Dietary calorie restriction is probably the most thoroughly investigated longevity promoting intervention since it was discovered that reductions of dietary intake, without malnutrition, could substantially increase lifespan in rats (McCay et al., 1935). Calorie restriction is suggested to extend lifespan by affecting the

intrinsic rate of ageing, and by suppressing pathogenesis (Colman et al., 2009, 2014; Hulbert et al., 2007a). Whether calorie restriction slows down the progress of ageing or delays the start at which the ageing process begins, remains an open question.

Increases in longevity have been observed using calorie restriction in many short-lived species such as the yeast *Saccharomyces cerevisiae* (Anderson et al., 2003; Guarente, 2005; Jiang et al., 2000; Lin et al., 2000; Sinclair, 2005), the nematode *Caenorhabditis elegans* (Klass, 1977; Walker et al., 2005), the fruit fly *Drosophila melagonaster* (Chapman and Partridge, 1996; Chippindale et al., 1993; Magwere et al., 2004; Moatt et al., 2016; Partridge et al., 2005; Simons et al., 2013) and in mammal models such as the mouse (Kim et al., 2002) and rat (McCay et al., 1935; Weindruch and Walford, 1988; Yu and Chung, 2001). Results from a recent seminal study on primates (rather than short-living species) suggest that calorie restriction does reduce age-related, and all-cause mortality in primates but has a fairly limited effect on lifespan (Colman et al., 2009, 2014; Mattison et al., 2017).

A consistent finding of calorie restriction studies is that there is a reduction in oxidative damage (Dubey et al., 1996; Hulbert et al., 2007b; Jové et al., 2014; Matsuo et al., 1993; Sanz et al., 2005; Sohal et al., 1994) and this is thought to be the main cause of life extension. Although it is accepted that calorie restriction leads to an extension of lifespan, the mechanism of action of how calorie restriction affects the physiology and biochemistry affecting lifespan remains poorly understood.

### 1.4.2 Pharmaceutical agents

In 2009, the Institute on Ageing at the University of Florida released results that identified rapamycin as the first pharmacological agent to extend average and maximum lifespan in a mammalian species (Harrison et al., 2009). Rapamycin is a macrolide compound that was first isolated from the soil bacterium of *Streptomyces hygroscopius* as a potential antibiotic (Vézina et al., 1975). It has mainly been used to prevent the rejection of kidneys during organ transplants. The extension of lifespan in mammals by rapamycin has been confirmed by other studies that established an optimal dose (i.e.15-45 uM) that lead to a consistent extension of average lifespan (up to 25%) when applied either early on or late, in life in male or female mice (Fok et al., 2014; Harrison et al., 2009; Miller et al., 2014; Neff et al., 2013) and in genetically heterogeneous mice (Miller et al., 2011). Previously it has been

shown that rapamycin also increases the average lifespan, in a dose-dependent manner, of yeast *Saccharomyces cerevisiae* (Powers et al., 2006), the nematode *Caenorhabditis elegans* (Robida-Stubbs et al., 2012) and fruit fly *Drosophila melanogaster* (Bjedov et al., 2010). The mechanisms by which rapamycin increases lifespan in the different models remains largely unresolved, but it is suggested that rapamycin inhibits TOR (target of rapamycin). TOR is an evolutionary conserved key protein pathway that was first discovered in yeast *Saccharomyces cerevisiae* (Heitman et al., 1991). The TOR pathway regulates various processes including cell growth, proliferation and metabolism in response to nutrients, growth factors, and stress (Abraham, 2002; Heitman et al., 1991; Oldham and Hafen, 2003). Interestingly, inhibition of the TOR pathway has been shown to extend lifespan in these same species; yeast (Kaeberlein et al., 2005; Powers et al., 2006), nematode (Jia et al., 2004; Vellai et al., 2003) and *Drosophila* fruit fly (Kapahi et al., 2004).

Another molecule widely studied in the context of ageing is resveratrol. Resveratrol is a natural phenolic product of plants produced in response to various stressors and is found in 'blue' fruits such as grapes, blueberries, and mulberries, and in red wines. The main interest of the research on this compound was originally started by the "French paradox", a high saturated-fat diet in the French population combined with a moderate consumption of red wine which seems to produce improved cardio-vascular outcomes (Catalgol et al., 2012; Hubbard and Sinclair, 2014; Renaud and de Lorgeril, 1992). Originally, resveratrol gained much attention for its antioxidant properties, inhibiting copper catalysed low-density lipoprotein oxidation (Frankel et al., 1993) and reducing lipid peroxidation in microsomes (Blond et al., 1995). It was therefore seen as a potential compound for the scavenging of free-radicals (Cao et al., 2003; Pervaiz, 2003; Queiroz et al., 2009). Resveratrol was found to consistently extend lifespan in a number of species including yeast *Saccaromyces cerevisiae* (Howitz et al., 2003; Jarolim et al., 2004; Yang et al., 2007), the nematode *Caenorhabditis elegans* (Greer and Brunet, 2009; Gruber et al., 2007; Wood et al., 2004; Ye et al., 2010; Zarse et al., 2010) and to some extent in fruit fly *Drosophilla melanogaster* (Chandrashekara and Shakarad, 2011; Wood et al., 2004). Resveratrol has also been shown to extend lifespan in social insects. Worker honey bees fed a 5% pollen-based diet supplemented with 130 µM of resveratrol increased their average lifespan by 33% compared to workers fed a 5% pollen-based diet (Rascón et al., 2012). Resveratrol has also been shown to increase average and maximum lifespan in the vertebrate killifish *Notrobranchius furzeri* (Valenzano et al., 2006)

but has shown no effects on the average lifespan of mice (*Mus musculus)* and rats (*Rattus norvegigus*) fed standard diets (Baur et al., 2006; da Luz et al., 2012; Miller et al., 2011; Pearson et al., 2008; Strong et al., 2013).

The mechanism of the action of resveratrol remains to be elucidated, but evidence suggests that resveratrol may extend lifespan through its antioxidant properties and the activation of sirtuin domain proteins (Bhullar and Hubbard, 2015; Howitz et al., 2003; Hubbard and Sinclair, 2014; Pervaiz, 2003; Wood et al., 2004). The mechanisms proposed could also act as a mimic of calorie restriction (Bhullar and Hubbard, 2015). In recent years, resveratrol has been speculated to improve metabolism, reduce inflammation and reduce the risk of age-related diseases, such as cancer, cardiovascular, and neurodegenerative diseases (Bhullar and Hubbard, 2015; Hubbard and Sinclair, 2014; Vaiserman et al., 2016). However, evidence from human trials and animal models remains inconclusive, and there is no evidence that resveratrol affects life expectancy in humans (Pallauf et al., 2016).

All in all, the biological processes that determine longevity remain to be discovered (Walker et al., 2005) and the evidence for pharmaceuticals and calorie restriction extending lifespan remains modest. The extension of lifespan by experimental manipulation appears to involve several conserved pathways among animal models including target of rapamycin (TOR; Evans et al., 2011; Kenyon, 2005), insulin/ insulin-like growth factor-like signalling (Bartke, 2005; Giannakou and Partridge, 2007) and sirtuins (Hubbard and Sinclair, 2014; Morselli et al., 2010; Wood et al., 2004). These pathways are thought to act on various transcription factors that induce metabolic changes and protective enhancements to reduce the accumulation of age-related damage (Fontana et al., 2010; Sinclair, 2005). Using a variety of experimental approaches, scientists have pinned down precise details about the regulation of these processes, the potential interaction between pathways, and their possible implication for ageing. We know what is going on, protein-by-protein, and gene-by-gene, as well as their interactions, yet we are missing the wood for the trees, as ageing remains largely unresolved. The results of this research, suggest that extending lifespan, and consequently delaying ageing, is possible but limited (e.g. maximum lifespan extension in rodents by calorie restriction is 50%). The extension in lifespan is, however, trivial compared to the tremendous variation in maximum lifespan among species, and in some cases within the same species (i.e. social insects). Thus, understanding natural variances in the lifespan of animals may

provide better insight into the mechanisms that underpin the processes of ageing.

### 1.4.3 Role of exercise in ageing

Perhaps one treatment that might significantly influence on ageing, as well as general well-being benefits, is physical exercise. The benefits of exercise on ageing remain largely speculative, but the benefits of exercise on heart conditions have been documented for decades. Given that the human population is ageing in general and thus raising average life expectancy, a growing challenge has been to make older people physically active and functionally independent with ageing. As well as affecting the nine hallmarks describe in Figure 1.1, ageing also correlates with several cardiovascular, cardiorespiratory, musculoskeletal, metabolic, and cognitive impairments (Garatachea et al., 2014). Interestingly, physical exercise has potential anti-ageing properties at multilevel system including i) increasing neurogenesis, ii) decreasing blood pressure and increasing maximal cardiac output as well as regional blood flow iii) improving respiratory function by increasing ventilation and gas exchange and iv) improving muscle strength and endurance (Garatachea et al., 2014; Nelson et al., 2007). Additionally, physical exercise has a positive anti-ageing impact at cellular levels on ageing hallmarks. An interesting influence of physical exercise is on telomere attrition. Ageing induces DNA damage accumulation, especially in some particularly sensitive chromosomal regions such as telomeres. Telomere length also decreases as cell age and has been proposed as a biological marker of ageing (Müezzinler et al., 2013). Physical exercise is associated with an upregulation of protective proteins (such as telomeric repeat-binding factor 2) and DNA repair pathway proteins as well as downregulation of negative regulator proteins of cell cycle progression in human (Werner et al., 2009). Aerobic exercise can also induce the transcription of gene encoding telomere-stabilizing proteins and telomerase activity in animals (Werner et al., 2008, 2009; Wolf et al., 2011) and also in human (Werner et al., 2009) via epigenetic mechanisms. Despite that the exact mechanisms on interaction remain to be elucidated, studies show that an important modulation of exercise exists on the epigenetics mechanisms, particularly in DNA methylation (Ling and Rönn, 2014). Exercise also acts as a stimulus for the migration/proliferation of the stem cell subsets from their home tissue to impaired tissues for regeneration (Tomaru et al., 2012). Aerobic exercise also induces systemic mitochondrial biogenesis as well as multi-organ oxidative capacities in human (Safdar et al., 2011), which suggests that physical exercise may help to

maintain a pool of bioenergetically functional mitochondria. Overall, that evidence emphasise that more effort should be devoted to gaining insights into molecular mediators of the benefits of exercise and implement effective exercise as interventions rather than searching for novel pharmaceutical targets of the ageing process.

# 1.5 Theories of ageing

The diversity of theories of ageing have increased drastically in the last decade and the number is now listed in the hundreds (Viña et al., 2007). This number is predicted to increase further as recent discoveries of molecular and cellular pathways are likely to lead to new theories of ageing (e.g. $NAD^+$ and Sirtuin; Li et al., 2017).

### 1.5.1 Central paradigm of the theories of ageing

The different variants of theories of ageing revolve around the damage cause by free radicals on macromolecules (termed oxidative stress) as well as the trend to accumulate damages with ageing. Free radicals can be produced from oxygen (reactive oxygen species), nitrogen (reactive nitrogen species) and sulfure (reactive sulfure species). However, the main free radicals are reactive oxygen species (ROS) that include a number of unstable molecules like superoxide anion ($O_2^{-\bullet}$), hydroperoxyl radical ($HO_2\bullet$), hydroxyl radical ($^\bullet OH$), nitric oxide (NO) and other species like hydrogen peroxide ($H_2O_2$) and singlet oxygen ($^1O_2$; Halliwell and Gutteridge, 2007). The balance between the production and neutralization of ROS is very delicate, and if the balance tends to the overproduction of ROS, the cells start to suffer the consequences of oxidative stress (Wiernsperger, 2003). Level of ROS is controlled by antioxidants that delays, remove or prevent oxidative damage to a target molecule (Halliwell, 2007). The reaction leading to the production of ROS is detailed in Figure 1.5-A. ROS are produced as normal part of metabolism in mitochondria through xanthine oxidase, peroxisomes, inflammation process, phagocytosis, arachidonate pathways, ischemia and physical exercise (Di Meo et al., 2016). From Figure 1.5-A, it can be seen that the production of ROS involve a number of reactions including various intermediates. Briefly, hydroperoxyl radical produced in mitochondria dissociate into superoxide radical ($O_2^{-\bullet}$) at pH 7.4. This extremely reactive molecule can interact with a number of molecule to generate ROS directly or through enzymes or metal-catalysed processes. Lipid radicals are also generated in the cascade reaction and will be discussed furthermore in section 1.5.2. Superoxide can be

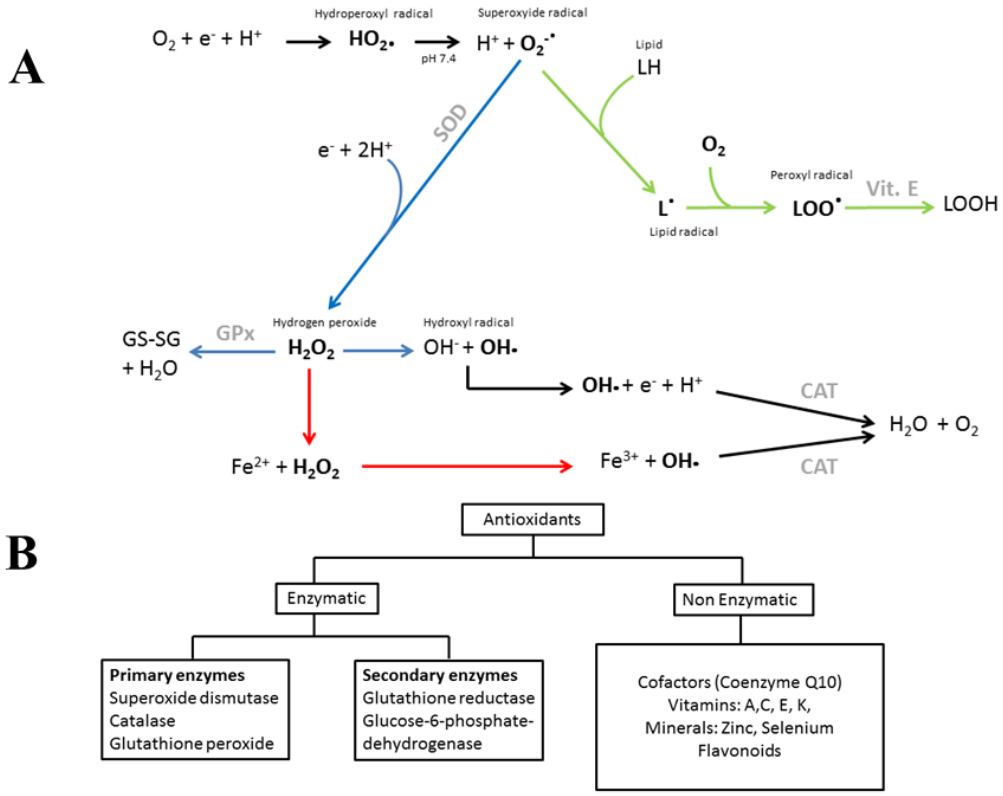


Figure 1. 5. A- Different reactions leading to the production and detoxification of reactive oxygen species (ROS). Green arrows represents lipid peroxidation describes in more details in Figure 1.9. Blue arrows represent the Haber-Weiss reactions, the red arrows represent the Fenton reactions. Radicals are depicted in bold while antioxidants are depicted in grey. SOD refers to the enzyme superoxide dismutase, CAT refers to the enzyme catalase while GPx refers to the enzyme glutathione peroxidase. B- Different classes of natural antioxidants. Enzymatic antioxidants are separated in primary and secondary enzymes.

detoxified to hydrogen peroxide ($H_2O_2$) through super oxide dismutase (SOD) through the Haber-Weiss reaction and finally to water by the catalase (CAT) enzyme. Hydrogen peroxide can reacts with iron catalyst (e.g. $Fe^{3+}$), which lead to the Fenton reaction and resulting in hydroxyl radical ($OH^{\bullet}$) that is neutralized into water. The production of ROS needs to be tighly regulated given the damage they can create on macromolecules and this is the role of antioxidants that neutralize ROS. The grand scheme of antioxidants can be descripted in 2 main groups; enzymatic and non-enzymatic antioxidants (Figure 1.5-B). The enzymatic antioxidants can be further characterized into primary and secondary enzymatic antioxidants with the primary consisting of three main enzymes: superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). SOD converts superoxide anions into hydrogen peroxide ($H_2O_2$) through the Haber-Weiss reaction (see details in Figure 1.5-A; Asayama and Burr, 1985; Tyler, 1975). Although hydrogen peroxide is not a radical, it is rapidly converted by Fenton reaction into hydroxyl radicals ($OH^{\bullet}$) which is very reactive. CAT converts hydrogen peroxide into water and molecular oxygen while glutathione peroxidase (GPx) neutralizes hydrogen peroxide into selenol (GS-SG) and therefore eliminates hydrogen peroxide as substrate for the Fenton reaction (see details in Figure 1.5-A; Deisseroth and Dounce, 1970; Mills, 1957). The secondary enzymes do not neutralize free radical directly but have rather supporting roles for other endogenous antioxidants. Glutathione reductase reduce glutathione from its oxidized form (i.e. allowing constant recycling of glutathione reductase) to allow continuing activity of glutathione peroxidase. Another enzyme, glucose-6-phosphate dehydrogenase is a cytosol enzyme involves in the pentose pathway and is involve in a cascade of antioxidant mechanism to prevent oxidative damage (Ratnam et al., 2006). In contrast to the relatively small numbers of enzymatic antioxidants, the non-enzymatic antioxidants are quite numerous and include enzyme cofactors, various vitamins, minerals and flavonoids that neutralize free radicals using various mechanisms (Halliwell and Gutteridge, 2007). Other larger molecules (e.g. plasma albumin), when damaged by ROS, can be considered incidental antioxidants (Peters, 1996). The complex picture emerging from the interaction between free radicals and antioxidants bring an important property that antioxidants must have. In order to reduce level of oxidative damage, antioxidants must have the ability after scavenging radical, to form a new radical that is stable from further oxidation (Halliwell and Gutteridge, 2007). Otherwise, antioxidants can become themselves free radicals that propagate oxidative damage. As previously mentioned, this could be achieved

through specific enzyme activity such as glutathione reductase or by combining different enzymes in the pathway, for example the combination of SOD with CAT and GPx to neutralize superoxide into water and molecular oxygen. Another example is the regeneration of vitamin E through Vitamin C (Turunen et al., 2004). Up to date, the oxidative stress theory of ageing remains the most accepted theory within the scientific community and the public, as evidenced by supplement stores filled with antioxidants.

### 1.5.2 Oxidative stress theory of ageing

The origin of the oxidative stress theory of ageing goes back to nearly 400 years B.C. as Aristotle proposed that the cause of aging could be due to the "fire of respiration" (Barnes, 1984). The first scientific observation as to why some mammals live longer than others probably came from Aristotle as he noted that some larger mammals have a longer lifespan compared to smaller mammals but also noticed some exceptions (i.e. lifespan of humans is longer compared to the lifespan of horses).

The observations by Aristotle were confirmed two millennia later by Max Rubner who noted that the lifespan of five mammal species (guinea pigs, cats, dogs, horses, and cows) increased proportionally with an increase in body size, and that the lifetime mass-specific metabolic rate was similar between all these species (Rubner, 1908). From these observations, and a series of experiments on *Drosophila melanogaster*, it was thought that senescence and ageing framed lifespan in terms of metabolic capacity (i.e. a finite number of heart beats, chemical transformations, a finite amount of oxygen consumption) and once that number was reached, mortality occurred. This was originally proposed by Rubner (Rubner, 1908) and later revisited by Raymond Pearl (Pearl, 1928). This theory, known as the rate of living theory, predicts that an increased metabolic rate per gram of tissue correlates with a shorter lifespan, and gained popularity in the saying "Live fast, die young."

However, this theory was eventually contradicted by multiple examples of size-matched and metabolic-rate matched animals with different lifespans, e.g. rats/pigeons (Barja, 1998), bats/mice (Prothero and Jürgens, 1987) and deer mice/lab mice (Ungvari et al., 2008). It was later demonstrated that metabolic by-products such as reactive oxygen species (ROS) were the main causative agents of ageing and potentially determinant of maximum longevity (Barja, 2007; Finkel and Holbrook, 2000; Ku et al., 1993; Lambert et al., 2007;

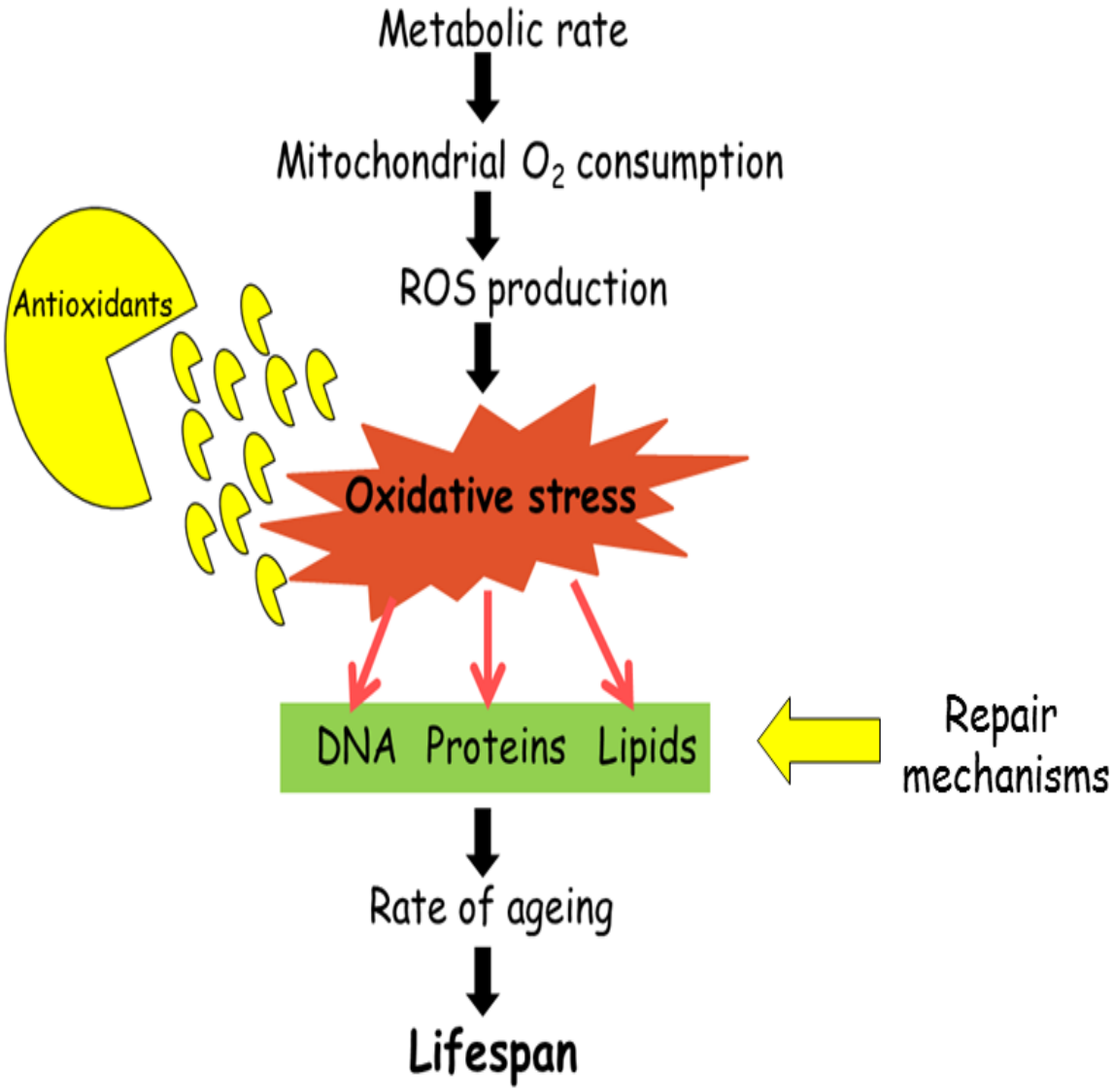


Figure 1. 6. Schematic summary of the framework of the oxidative stress theory of ageing. Metabolic rate is mainly driven by mitochondrial respiration. Mitochondria consume oxygen and simultaneously produce reactive oxygen species (ROS), that generate oxidative stress if ROS levels are not balanced by endogenous antioxidants. Oxidative stress damages biological molecules such as DNA, proteins, and lipids. The accumulation of damaged biological products influences the rate of ageing and therefore lifespan of organisms. This figure is adapted from Hulbert et al. 2008.

Mookerjee et al., 2010). The proposed metabolism-lifespan link led to the formulation of a new theory known as the free-radical theory of ageing, first proposed by Harman (Harman, 1956), and currently referred to as the oxidative stress theory of ageing (Figure 1.6; Beckman and Ames, 1998; Hulbert, 2008) and redox stress hypothesis (Sohal and Orr, 2012). The theory proposes that the products of metabolism react with oxygen to form free radicals (e.g. ROS) that can then react with other molecules that can become toxic and damage macromolecules (a process termed oxidative damage; Gutteridge, 2007). The oxidative stress theory of ageing proposes that it is the interplay of free radical production from metabolism (generators), a lack of adequate defence (mechanisms of maintenance/repair, enzymes, antioxidants) against the radicals as well as the fatty acid environment (propagation) that influence the pace of ageing, and ultimately, lifespan (Figure 1.6). Accordingly, the lifespan of birds and mammals has been shown to be inversely correlated with oxidative damage (Barja, 1998; Pamplona et al., 1998). The oxidative stress theory of ageing proposes that the accumulation of oxidative damage, particularly in mitochondria, is the main proximate cause of ageing, and that lifespan is determined by the rate at which this damage occurs.

### 1.5.3 The membrane pacemaker theory of ageing

A modification of the oxidative stress theory of ageing, "The membrane pacemaker theory of ageing," suggests that membrane fatty acids and particularly polyunsaturated fatty acids (PUFA) are an important determinant of maximum lifespan (Figure 1.7; Hulbert et al., 2005). The reason PUFA may influence longevity is that these fatty acids are readily incorporated into membrane phospholipids (Abbott et al., 2010; Hulbert et al., 2014; Kelly et al., 2014) and are highly oxidisable (i.e. autoxidisable). PUFA possess methylene groups (i.e. $CH_2$) between their double bonds (e.g. =CH-**$CH_2$**-CH=) that are commonly referred to as *bisallylic* methylene (see details in Figure 1.8). These methylenes have hydrogen with weakened bond energies (due to the attraction of their electrons toward the double bonds on either side) making them susceptible to removal by free radicals. In contrast, monounsaturated fatty acids (MUFA; with one double bond) have *allylic* methylene (Figure 1.8) on either side of their single double bond with stronger hydrogen bond energies making them less susceptible to lose to radicals. Saturated fatty acids (SFA, with no double bond) with arrays of methylene have hydrogens that are strongly held to their carbon centers and are very resistant to radical abstraction.

The weakened bond energies of hydrogen on *bisallylic* methylene make PUFA up to a 1000-fold more susceptible to radical 'attack' compare to SFA and MUFA (Else and Kraffe, 2015). The loss of hydrogen (with its electron) results in a carbon-centered radical (L•) on the fatty acid chain that binds oxygen (if available) to form a lipid peroxyl radical (LOO•). This new radical seeks a further hydrogen atom commonly provided by another *bisallylic* bond on the same PUFA molecule or from surrounding PUFA molecules causing propagation of the reaction. The presence of lipid radicals (L•), lipid hydroperoxides (LOOH) plus other products formed as part of the breakdown of lipid hydroperoxides such as alkanals, alkenals, hydroxyalkenals, glyoxal, and aldehydes can result in damage to surrounding macromolecules (Esterbauer et al., 1991; Gutteridge, 2007). This reaction sequence is often referred to as peroxidation because a peroxyl radical (LOO•) is formed as part of the oxidative process (Figure 1.9). This autocatalytic process, once initiated, can be stopped by quenching via antioxidants or by other processes such as self-annihilation, substrate limitation or specific enzyme activity.

Biological membranes are made up of dozens of phospholipid molecules (Abbott et al., 2013; Cortie et al., 2015; Mitchell et al., 2007) and thus membranes contain a variety of SFA, MUFA and PUFA. Once the fatty acid composition of a membrane is characterised, it is possible to estimate its relative capacity to undergo peroxidation. This dimensionless number (referred to as membrane peroxidation index or membrane PI) is calculated by summing the relative proportion of the different PUFA (expressed as % of total fatty acids) multiplied by the factor indicating their capacity to peroxidise:

$$\text{Peroxidation Index (PI)} = (\sum \%\,\text{di} - \text{PUFA} * 1) + (\sum \%\,\text{tri} - \text{PUFA} * 2) + (\sum \%\,\text{tetra} - \text{PUFA} * 3) + (\sum \%\,\text{penta} - \text{PUFA} * 4) + + (\sum \%\,\text{hexa} - \text{PUFA} * 5).$$

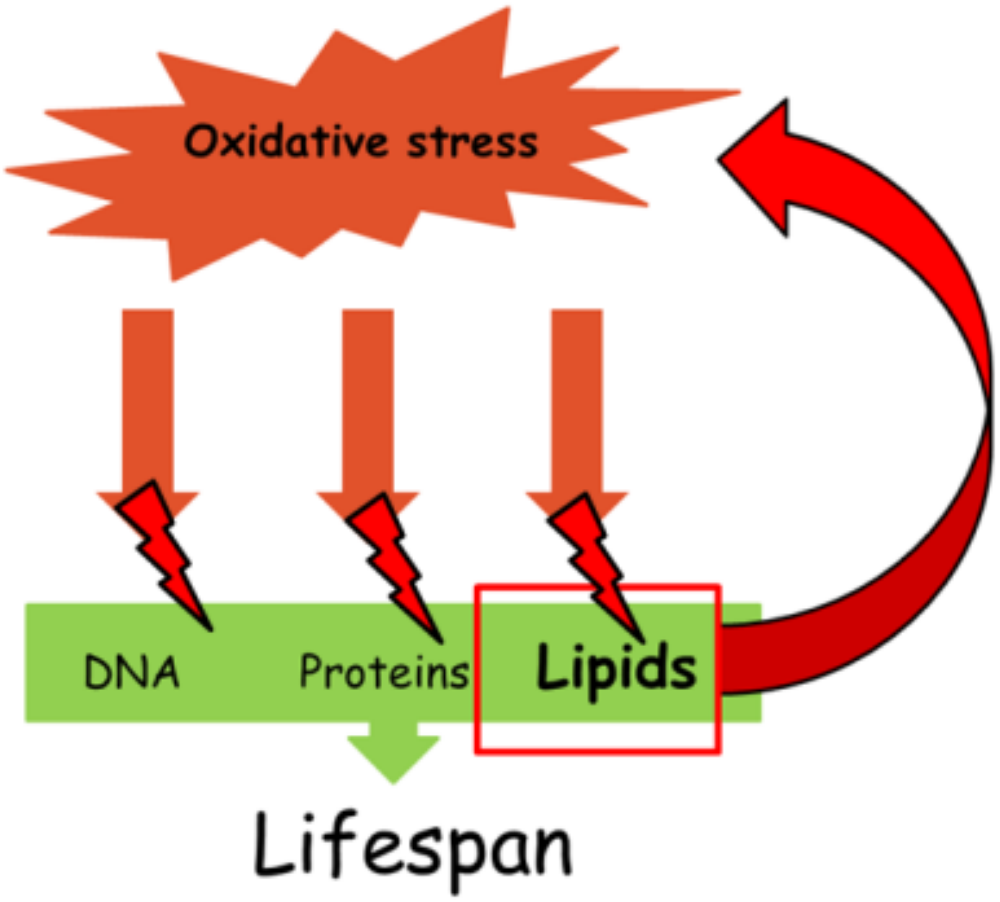


Figure 1. 7. Schematic summary of the membrane pacemaker theory of ageing. The membrane pacemaker theory of ageing suggests that oxidative stress on membrane lipids will produce secondary based ROS, which will lead to further oxidative stress through an auto chain reaction (normally termed lipid peroxidation and illustrated with red arrows) to other macromolecules. The more polyunsaturated the membranes, the more damage potentially produced. Adapted from Hulbert, 2005.

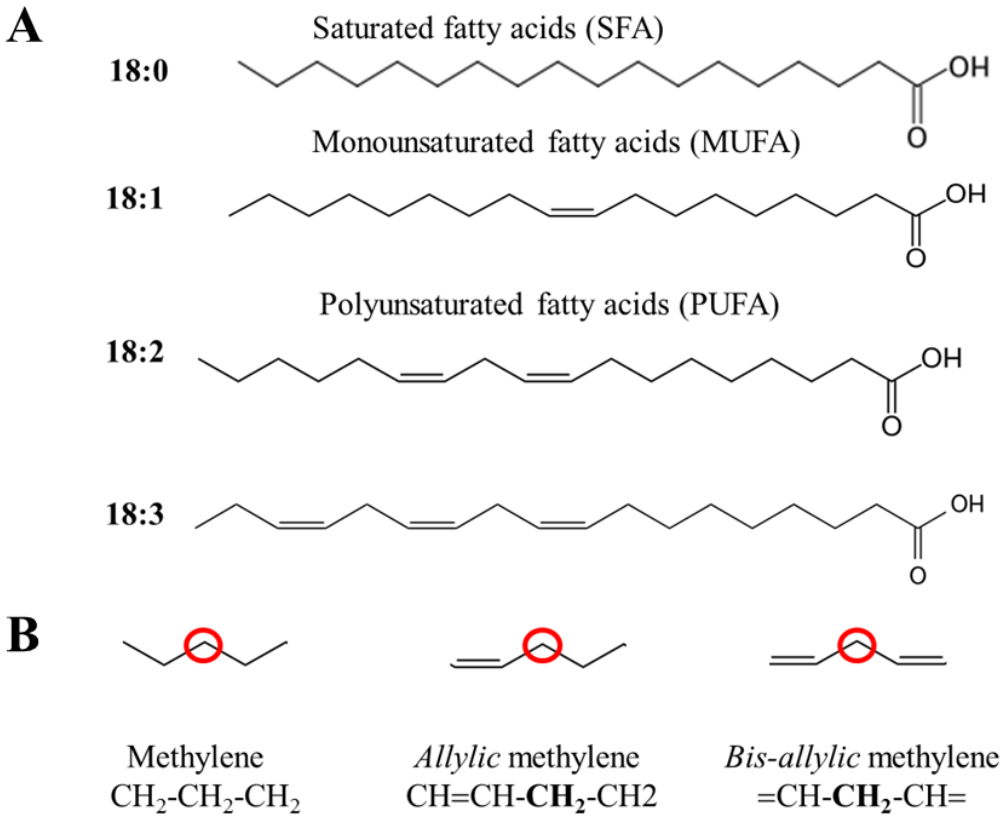


Figure 1. 8. A) Structure of the three main types of fatty acids: saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). In this particular example, all fatty acids are 18 carbons long. The SFA is 18:0. The MUFA is 18:1n-9 and the PUFA are 18:2n-6 and 18:3n-3, respectively. B) Structure of the different methylene groups with their chemical composition, red circle indicates the relevant methylene. SFA contain only methylene groups (i.e. $CH_2$-$CH_2$-$CH_2$), MUFA contained two *allylic* methylene (i.e. -$CH_2$-CH=CH-$CH_2$-$CH_2$) groups located on either side of the double bond while PUFA contain multiple *allylic* methylene groups and at least one *bis-allylic* (i.e. =CH-*$CH_2$*-CH=) depending on the degree of polyunsaturation. In this particular example, 18:2 contains one *bis-allylic* and 18:3 contains two *bis-allylics*.

Empirical experiments of oxygen consumption during peroxidation of different PUFA have shown that the more polyunsaturated the PUFA, the greater its rate of oxygen consumption and therefore the more lipid peroxidation product produced. The relative oxygen consumption of individual PUFA has been used to calculate factors that indicate the relative peroxidative potential of individual PUFA (originally by Holman, 1954 and revised by Cosgrove et al., 1987). For instance, linoleic acids 18:2 which has two double-bonds and therefore one *bis-allylic* $CH_2$ is given a factor of "1'' while linolenic acid (18:3) with 3

double-bonds and therefore 2 *bis-allylic* $CH_2$ has a factor of "2". Experimentation has also confirmed that SFA and MUFA, which lack *bis-allylic* $CH_2$ bonds, do not undergo significant lipid peroxidation (Else and Kraffe, 2015).

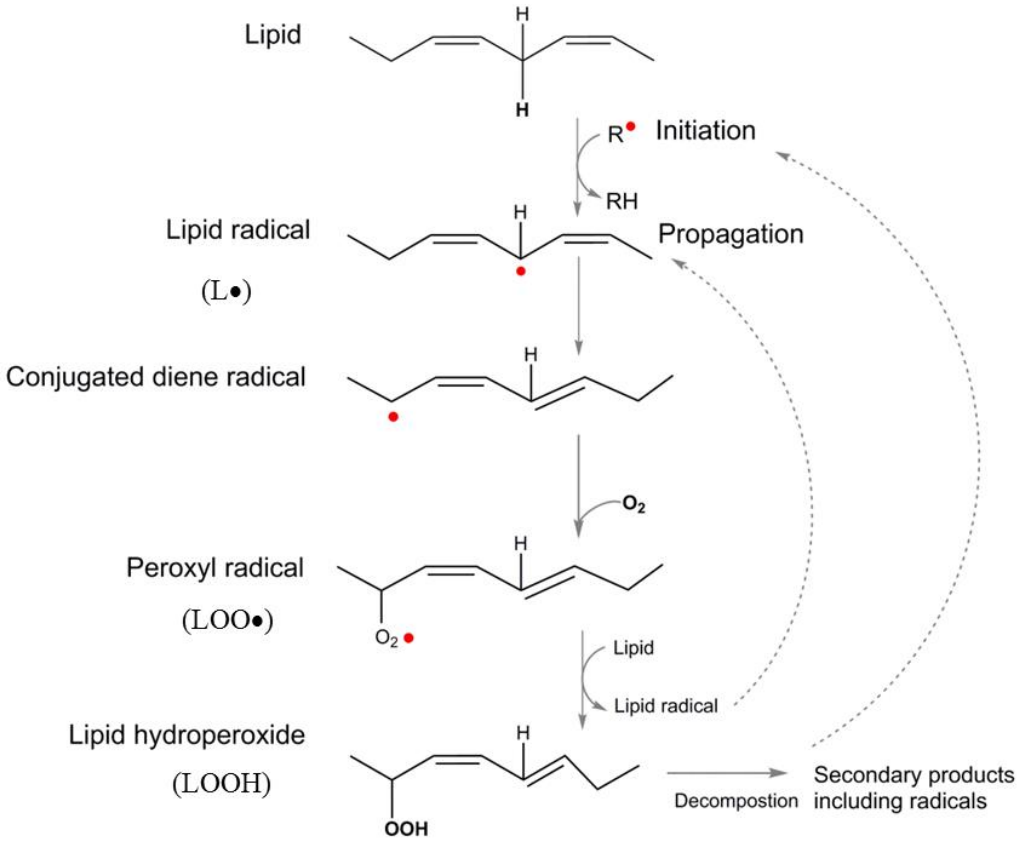


Figure 1. 9. Schematic summary of the mechanism of the lipid peroxidation. Lipid peroxidation has three distinct phases: initiation by a free radical, propagation where the rate of lipid peroxidation increases exponentially, and termination where the reaction runs out of substrate or is stopped by antioxidants. Secondary products made during lipid peroxidation include alkanals, alkenals, hydroxyalkenals, glyoxal, and aldehydes that can produce further damage to surrounding macromolecules. Adapted from Cortie, 2015.

The first evidence that leads to the formulation of the "membrane pacemaker theory of ageing" came from the observation that membrane fatty acids of several tissues (i.e. liver, kidney, muscle, and heart) of different-sized mammals vary in their composition in a systematic manner. Small mammal species have membranes highly polyunsaturated with a

low content of MUFA, while larger mammals have membranes highly monounsaturated with a low content of PUFA (Couture and Hulbert, 1995; Hulbert et al., 2002). A series of studies published at around the same time demonstrated that the mitochondrial membrane peroxidation index correlated with the maximum lifespan of different-sized mammals (Pamplona et al., 1998, 2000a, 2000b). These findings were later correlated with the varying membrane compositions of various species that showed that the peroxidation index of the membranes of various tissues and organelles of mammals is inversely correlated to their maximum lifespan (Hulbert, 2010). Birds for example that are similarly sized to mammals, but can be much longer living showed a similar relationship between the membrane peroxidation index and longevity (Hulbert et al., 2014). The same relation between membrane fatty acid composition and longevity has also been reported for some invertebrate species such as bivalve molluscs (Munro and Blier, 2012), the nematode worm *Caenorhabditis elegans* (Shmookler Reis et al., 2011) and also female honey bees (Haddad et al., 2007). Honey bees have been suggested as a good model organism to investigate the mechanisms of ageing because there is extraordinary variation in lifespan between genetically identical female castes (Keller and Genoud, 1997; Keller and Jemielity, 2006).

## 1.6 Eusocial insects

Interestingly, honey bees were once considered as vertebrates in the early 1800s following a remarkable comparison made by the apiarist Johannes Mehring (1815-1878). Her main argument was that a colony is a single whole organism or super-organism. Worker bees represent the body organs required for digestion and maintenance while the queen (female and highly reproductive) and drones (male caste) represent the genital organs. Honey bees are invertebrates, but the term ‘super-organism’ persists in association with these social insects.

Honey bees are part of the eusocial insects that are divided into two orders: *Isoptera* (termites) and *Hymenoptera* (ants, wasps and bees). Eusociality represents the highest level of sociability in animals and is described by having a cooperative care of young, overlapping generations within a colony of adults, a hierarchy mainly controlled by a few individuals, if not a single individual, and a division of labour into reproductive and non-reproductive groups (Michener, 1974; Wilson, 1971). Eusociality is also found in two species of mammals, the naked mole rat (*Heterocephlus glaber*) and the Damaraland mole rat

(*Fumomys damarensis*), and perhaps one species of crustacean, the sponge-dwelling shrimp (*Synalpheus regalis*; Duffy, 1996).

### 1.6.1 Different castes of the eusocial system

Eusocial animals live in a colony system, where different castes interact to maintain the colony. Queens and reproductive males have the responsibility of replenishing the colony while sterile female workers execute all tasks related to maintaining favorable conditions for the brood. In honey bees, the castes consist of the three different phenotypes: highly reproductive female queens, sterile female workers, and reproductive males (drones). Queens are bigger compared to workers and similar in size compared to drones. Drones have bigger eyes compared to the females, and don't have a 'stinger' apparatus.

Honey bee colonies typically contain 50,000 workers with only one queen and a limited number of males (i.e. 1-5% of the population; Boes, 2010; Free et al., 1975). In honey bees, queens are made, not born. The same diploid egg (2*n*) can either become a worker or a queen depending on the size of the cell, the quantity of food fed to the larva, and the social context in the hive (Haydak, 1970; Tautz, 2008; Winston, 1987). Within a hive, female adults (whether queen or worker)) will also have identical genome as they share the same father. The queen decides to fertilise (2*n*), or not (1*n*) every single egg she lays over her lifetime. Interestingly, this behavior will lead to the possibility that thousands of workers will have identical genome to sister queens as the mother queen will use the sperm from the same father to fertilize thousands of eggs that could become either queen or worker. A haploid egg (1*n*) laid in a special cell (i.e. slightly larger compared to the normal cells for workers) results in a male phenotype. All larvae grow through similar stages, immersed in the same nutritive fluid (i.e. royal jelly) and emerge as adults after 18 to 24 days of development (see Figure 1.10 for details on developmental stages). During their first week as an adult, a queen will mate with several drones during her nuptial flights and then remain inside the hive for the rest of her life unless swarming to establish a new colony. A single queen can lay as many as 8 million eggs over her lifetime (Winston, 1987). Queens will only produce drones during a restricted period of the year (typically during the warmest months of the year), and workers expel drones when the colony prepares for winter (Hrassnigg and Crailsheim, 2005). Drones have the function of producing sperm, and of mating with a queen. Reproduction is suicidal for drones as queens tear their endophallus during copulation, and drones die shortly after

copulation.

Workers perform all tasks related to colony maintenance, as they transition through different life-history stages (a process termed temporal polytheism), from in-hive nurses to roving foragers. Nurse bees feed larvae, clean and build honeycomb cells, groom the queen, store incoming pollen, and feed newly emergent bees during the first weeks of their adult life. Foragers collect pollen, nectar, and water from the environment to provide for the colony (see Winston, 1987 for a full review on life-history stages). The duration of both stages is variable and adapted according to the needs of the colony (Remolina and Hughes, 2008). Another difference between castes is the type of food consumed after emergence as adults. Workers begin feeding gradually on pollen a few hours after emerging as adults, although they are also fed a liquid-fluid by older nurse bees for the first few days of their adult life to complete their development (Haydak, 1970; Hrassnigg and Crailsheim, 2005; Weaver, 1966; Winston, 1987). Adult workers have to ingest pollen and nectar to provide themselves with sufficient nutrients for their different roles in the adult stage. They digest proteins from the pollen to produce secretions (i.e. royal jelly) in their hypopharyngeal glands and feed larvae in comb cells. The consumption of pollen is higher in newly emerged workers compared to older workers. This change in pollen consumption is related to changes in physiology (e.g. atrophy of glands after workers start foraging) that could limit their ability to digest proteins and fats as they age (Loidl and Crailsheim, 2001; Moritz and Crailsheim, 1987). Worker bees consume most of the pollen in the form of 'bee bread', which consists of pollen, regurgitated nectar, honey, and glandular secretions. Bee bread also differs from fresh pollen in having a lower pH, less starch (Ellis and Jr, 2009; Jr and Shimanuki, 1978) and it has a higher nutritive value compared to collected pollen (Jr and Shimanuki, 1978; Pernal and Currie, 2000). When workers start foraging, they reduce their pollen consumption and shift their diet toward honey as they use carbohydrate as an exclusive substrate when flying (Suarez et al., 2000, 2005).

Adult queens are fed a liquid-fluid (assumed to be the same royal-jelly that they received as larva) their whole life and do not eat pollen (Haydak, 1970; Tautz, 2008; Winston, 1987). This difference in diet between queens and workers as adults leads to differences in the membrane lipid composition between the female castes (discussed later). Little information is available regarding the diet consumed by adult drones. Drones consume less pollen compared to workers, and most pollen consumption appears to take place during

the first few days after emergence as an adult (Szolderits and Crailsheim, 1993a). Drones also rely on workers to feed them royal jelly. It has been demonstrated that isolated drones fed pollen without nursing bees (and therefore no jelly feeding by workers) do not fully develop their mucus gland, which is required to enable a strong connection during mating (Koeniger and Koeniger, 1991; Mindt, 1962; Szolderits and Crailsheim, 1993a). Drones also have a limited crop capacity compared to workers (Mindt, 1962; Snodgrass, 1985; Winston, 1987) that suggests drones are at a higher risk of starvation by depleting their energy reserves on homing flights.

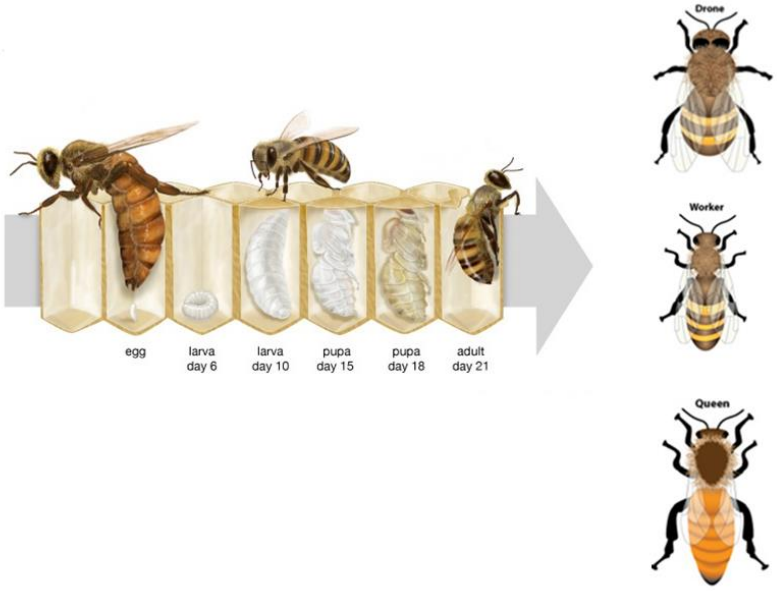


Figure 1. 10. Various stages of development in honey bee. The queen lays an egg into a clean cell. Larva emerging from the egg is fed royal jelly by nurse worker bees for their first four days of development. After four days, worker bees seal the cell, and the larva pupates into an adult bee (pupation). The adult worker bee emerges from a regular size cell around 21 days after the queen laid the egg. Drones take 24 days to complete their development, and the egg needs to be laid into a bigger cell compared to worker cells. Queens are the fastest to develop and only take 18 days to emerge from a special cell that is much bigger compared to the worker and drone cells. All larvae are fed royal-jelly, irrespective of their final phenotype. Up to the third day, all female larvae are bi-potent and can become either workers or queens. However, after three days, future queens receive more food compared to workers and drones (Haydak, 1970; Winston, 1987).

### 1.6.2 Longevity of the different castes

One of the most intriguing differences in eusocial insects is the difference in longevity between the castes. Queens achieve an order of magnitude longer lifespan compared to workers. For instance, queen ants can live up to 28 years (Haskins and Haskins, 1992) while workers usually live for 3-4 years (reviewed in Keller, 1998). Termite queens are known to live for decades while workers also live for 3-4 years (Keller, 1998; Lucas et al., 2016; Page and Peng, 2001). In honey bees, queens can live for several years (longest lifespan recorded being eight years; Wilson, 1971), while drones and workers normally live for 4-6 weeks (Page and Peng, 2001; Winston, 1987; Winston et al., 1981). This 100-fold difference in lifespan between genetically identical queens and workers makes honey bees an ideal model to investigate the process of ageing.

### 1.6.3 Mechanisms to explain the difference in lifespan between queens and workers.

Despite the iconic value of honey bees in the natural environment and to the economy of commercial crops, very few studies have compared queens and workers to elucidate the extraordinary difference in lifespan. Even less information is currently available regarding the lifespan of drones (Rueppell et al., 2005). Most studies looking at honey bees have described changes in behaviour, anatomy, and physiology on short-lived workers and speculated on the relevance of the findings to explain the long lifespan of queens. Furthermore, most previous studies have focused on establishing the importance of extrinsic factors (predation, environmental conditions) and behaviour (nurse versus foragers) to explain the difference in lifespan between the short-living workers and long-living queens. Of course, this could partly explain the lifespan difference between the castes as the queen is protected from predation and most pathogens inside the nest. However, even when worker bees are prevented from foraging, their lifespan remains shorter compare to queens (Hsieh and Hsu, 2013; Manning et al., 2007; Remolina et al., 2007) and the cause of this premature death remains poorly understood. To date, no study has compared the different castes over their whole life cycle (i.e. from larva to old adults), and few studies have compared age-matched workers and queens in an experimental design to gain insights into the difference in ageing between the castes.

Using the oxidative stress theory of ageing, predictions can be made that: i) long-lived

queens would have lower oxidative damage compared to short-lived workers and ii) long-lived queens would possess increased mechanisms for repair and protection against oxidative damage compared to workers. As previously discussed, research on mechanisms that could mediate lifespan determination in social insects is still in its infancy, as only a few research groups have tackled ageing related questions using social insects. Results from this research remain largely inconclusive and sometimes show conflicting evidence from the predictions of the oxidative stress theory of ageing.

Queen longevity appears not to be based on increased antioxidant gene expression as eight major antioxidant enzymes show no difference between queen and worker honey bees (Corona et al., 2005). Interestingly, the level of gene expression of antioxidant enzymes decreases with age in queens whereas it is maintained, or even increased in workers (Corona et al., 2005). Ant queens (*Lasius niger*) also have lower antioxidant enzyme gene expression, as well as lower levels of enzyme activity of Cu-Zn-superoxide dismutase compared to female workers (Parker et al., 2004). Another study (Hsieh and Hsu, 2011a, 2011b) that compared various antioxidant enzymes in queen and worker honey bees showed contrasting results. Over a series of experiments, the research group compared various antioxidant enzymes and oxidative damage markers in trophocytes and fat cells in two-month-old queens (young queens) versus 16-month-old queens (Hsieh and Hsu, 2011b, 2013; Hsu and Lu, 2015) and one-day-old workers (young worker) versus 50-day-old workers (Chuang and Hsu, 2013; Hsieh and Hsu, 2011a; Hsu and Hsieh, 2014). Their result showed that the activities of most antioxidant enzymes (i.e. catalase, Mn-superoxide dismutase, Cu-Zn-superoxide dismutase, and glutathione peroxidase) increased in 16-month-old compared to two-month-old queens. This is unlike the situation in workers where the level of catalase and glutathione peroxidase increased in 50-day-old compared to one-day-old workers, whereas the activities of both superoxide dismutases (Mn and Cu-Zn) decreased with age. The higher antioxidant activity in 16-month-old compared to young queens was proposed as a mechanism used by queens to reduce oxidative stress in trophocytes and fat cells as they age. However, absolute levels of antioxidant capacity suggest otherwise, as the level of various antioxidants was between 2 to 10-fold higher in workers compared to queens, suggesting that workers have a much greater antioxidant capacity than queens.

Three markers of cellular senescence: malondialdehyde (i.e. a product of lipid

peroxidation), protein carbonyl, and lipofuscin pigments all increased with age in both workers (Hsieh and Hsu, 2011a) and queens (Hsieh and Hsu, 2011b). However, the two studies (Hsieh and Hsu, 2011a, 2011b) found that the levels of these ageing factors were either very similar between the castes or even higher in queens compared to workers. This suggests that these commonly used parameters of ageing may not be relevant to the difference in lifespan between the castes. The difference in lifespan between queen and worker ants is associated with differences in the expression of genes involved in DNA and protein repair (Lucas et al., 2016). The study by Lucas et al. revealed that the expression of genes involved in processes of DNA repair and the ubiquitin-proteasome system was higher in the legs and brains of two-month-old queens compared to age-matched workers (Lucas et al., 2016). However, the level of DNA damage (estimated as a percentage of DNA damage) was similar in one-year-old queens compared to one-year-old workers, for both tissues (Lucas et al., 2017). The same study also revealed that protein ubiquitination (i.e. a marker of oxidative damage to proteins) was also similar between one-year-old worker ants and one-year-old queen ants, and decreased with ageing (Lucas et al., 2017) in both leg and brain tissue.

Telomere lengths (another indicator of ageing) were also reduced when comparing male ants (*L. niger*) to female worker and queen ants but were not different between the two female castes (i.e. queens and workers; Jemielity et al., 2007). These findings are consistent in honey bees where telomere lengths are similar between queens and workers, regardless of age (Hsieh and Hsu, 2011a, 2011b).

Another mechanism that has been proposed to explain the difference in lifespan between the castes is vitellogenin. Vitellogenin is an egg yolk precursor protein produced in the females of nearly all oviparous species including fish, amphibians, reptiles, birds, and most invertebrates (Robinson, 2008). In honey bees, vitellogenin is synthesised in fat body cells and released in the hemolymph. In worker bees, vitellogenin is used to produce royal jelly (Amdam et al., 2003) and workers are also thought to transfer a vitellogenin precursor to queens through royal jelly (Engels, 1974). After adult emergence, the level of vitellogenin in queens is low, but soon after the first intake of food, the level increases dramatically to reach 70% of total protein in the haemolymph. The level of vitellogenin decreases after queens start laying eggs, but this decrease can be prevented in non egg-laying queens that interrupt egg

production (Engels, 1974), suggesting that vitellogenin is used for egg production in queens. The level of vitellogenin also increases in egg-laying workers, supporting the use of vitellogenin in egg production (Engels, 1974). In worker bees, juvenile hormone and vitellogenin show a unique inverse correlation (Münch et al., 2008) related to life-history stages of workers (Amdam and Omholt, 2003). The level of vitellogenin reduces after workers transit from in-hive nurses to foragers and is associated with atrophy of the hypopharyngeal glands (where the royal jelly is produced). The level of vitellogenin in foragers can be reversed to nurse level with social manipulation, with a concurrent reduction of juvenile hormone and an increase in the size of hypopharyngeal glands (Amdam et al., 2005). This change has been suggested to regulate worker immunity (Münch et al., 2008), as well as having potential antioxidant benefits (Amdam and Page Jr., 2005). Interestingly, the increase of vitellogenin in reverse worker is associated with an extension in lifespan (up to 6 weeks; Munch and Amdam, 2010). Diutinus or so-called 'winter bees', that survived up to eight months (Munch and Amdam, 2010) have a similar physiological profile compared to nurse bees, with well developed hypopharyngeal glands, low juvenile hormone titre, and high vitellogenin content (Fluri et al., 1982) and accordingly remain inside the hive for most of the winter. Overall, the extension of lifespan in reverse nurse workers and winter bees compared to summer worker bees is intriguing, but the lifespan of workers remains shorter (6 weeks to 8 months) compared to queens (average lifespan of 5.6 years; Keller and Genoud, 1997). The evidence for vitellogenin being involved in ageing are currently based on correlation rather than a cause-effect relationship. Furthermore, vitellogenin is used for different purposes in queens compared to workers, which may ultimately underlie the difference in vitellogenin content between the castes.

Perhaps one of the first characteristics documented in the honey bee system was the difference in diet between queens and workers during adult life (Haydak, 1970). One of the best trademarks associated with honey bees is probably this difference in diet. The exclusive consumption of royal jelly by long-lived queens has been used as an argument to promote the sale of derived royal jelly products for decades. Very surprisingly, the difference in diet between workers and queens has received very little attention from scientific circles until recently. One study has suggested that distinctive nutrition during adult life could explain differences in longevity between the female castes (Haddad et al., 2007). Up to adult emergence, both female castes are fed a liquid jelly. However, a major difference in the types

of food consumed by bees occurs following their emergence as adults. As previously mentioned, queens are fed 'mouth-to-mouth' a fluid by worker bees (assumed to be the same royal jelly that they receive as larvae) that is low in polyunsaturated fatty acids (PUFA). In contrast, following emergence, worker bees begin to consume honey and pollen in the form of 'bee bread' that is known to have a high PUFA content (Haddad et al., 2007; Manning and Harvey, 2002; Manning et al., 2007). For worker bees, this change in diet coincides with changes in their membrane phospholipids, with an increase in the proportion of PUFA and a decrease in the proportion of monounsaturated fatty acids (MUFA; Haddad et al., 2007). In queens, there is no change in the membrane fatty acid composition, which remains highly monounsaturated throughout life (Haddad et al., 2007; Robinson and Nation, 1970). This change in membrane composition leads adult worker bees to have membranes that are more susceptible to peroxidation (i.e. have a higher membrane PI) compared to queens that maintain membranes that are resistant to peroxidation (low membrane PI). Surprisingly, this hypothesis remains to be tested as one of the only mechanisms proposed to explain the difference in lifespan between female castes.

Another obvious difference between the castes lie in the behavior and more particularly in the amount of flight each caste experiences over their lifetime. Queens only fly during their nuptial flights and remain inside the hive for most of their life. It is assumed that the new queen will leave when a colony is swarming but strong evidence is still required to confirm this hypothesis. In contrast, workers will fly more compared to queens as they transit from nurse to forager and collect pollen, nectar and water to fulfill the needs of the colony. Although it is very tempting to stipulate that the difference in lifespan between the castes could be explained by the difference in flight activity over their lifetime as well as potential damage to wings, workers maintain in captivity do not live as long as queen. Workers maintained in cages, and fed pollen had an average lifespan between 23 to over 42 days (Manning et al., 2007; Pasquale et al., 2016; Wang et al., 2014). The longest lifespan in worker bees is achieved in winter when worker bees act a "heater" to maintain the colony alive during the coldest months of the year. During winter, worker lifespan increased drastically and workers can survive up to 8-months (Munch and Amdam, 2010). Interestingly, this extension of lifespan is correlated with a reduction of flights, given that the workers are restrain inside the hive during winter. A reduction of flight activity is associated to a 3-fold extension of lifespan in the housefly *Musca domestica*, a reduction in

mitochondrial $H_2O_2$ production and also correlate with a reduction in mitochondrial oxidative damage (Yan and Sohal, 2000). Even if this extension of lifespan will be directly transfer to honey bees, lifespan of workers will remain in months at best compared to queens can live for years. Overall, this suggests that wing damage may only contribute partially to shorter lifespan compared to the queens. Furthermore, that is also underestimating the large turnover of eggs production that the queen will produce over her lifetime (up to 2000 eggs a day at the highest peak of the summer) in contrast to sterile worker bees.

Overall, the results from the limited number of studies examining queens and workers suggest that the link between oxidative damage and repair mechanisms in social insects is more complex than usually recognised in flies, worms, and mammals, and could potentially go against some of the predictions of the oxidative stress theory of ageing (See Table 1.2).

### 1.6.4 Advantages of using honey bees as a model organism to study ageing

The honey bee caste is a good gerontological model because i) it is conditionally sterile, ii) it possesses a flexible conditional age determination system, and iii) it is represented in a wide range of habitats from tropical Africa to northern Europe suggesting that honey bees have adapted their physiology to different habitats (i.e. extreme winter conditions). The honey bee system is also very interesting from an evolutionary point of view. Whereas typical ageing research 'forces' animals to live longer through different treatments and interventions, honey bees provide a system that has naturally evolved to create different phenotypes that show a difference in lifespan of up to 100-fold. This system is also attractive since bees are produced in large numbers with individuals of uniform size and the same genetics allowing for controlled experiments at a high resolution, a feature not easily accessible in other organisms. Experiments with bees can specifically inform us about the sources of age variation in honey bees, which is a species of great economic and ecological importance. Finally, honey bees are post-mitotic organisms and therefore studying ageing in honey bees may involve cellular senescence.

Table 1.2. List of parameters involved in the oxidative stress theory of ageing with predictions and current observations in social insects.

| Parameter | Predicted result | Observed result | Reference |
|---|---|---|---|
| Metabolic rate | Queens will have a lower metabolic rate compared to workers | Maximum metabolic rates similar between queen and worker honey bees | Fahrenholz et al., 1992; Harrison, 1986; Harrison et al., 2005 |
| Mitochondrial oxygen consumption | According to the 'uncouple to survive theory' (Brand, 2000) to reduce ROS production, queen mitochondria might be more uncoupled compared to workers | NA | NA |
| ROS production | Queens will have a lower level of ROS compared to workers | Queen and worker honey bees have similar levels of ROS production | Hsieh and Hsu, 2013; Hsu and Hsieh, 2014 |
| Protein damage | Queens will have a lower level of protein damage compared to workers | Queen and worker ants have similar levels of protein damage | Lucas et al., 2017 |
| DNA damage | Queens will have a lower level of DNA damage compared to workers | Queen and worker ants have similar levels of DNA damage | Lucas et al., 2017 |
| Lipid damage | Queens will have a lower level of lipid damage compared to workers | Level of malonaldehyde (i.e. a lipid peroxidation product) are similar between queen and worker honey bees | Hsieh and Hsu, 2013; Hsu and Hsieh, 2014 |
| | | Queens have a lower membrane peroxidation index than worker honey bees | Haddad et al., 2007 |
| Antioxidants | Queens will have higher antioxidant levels compared to workers | Levels of gene expression and antioxidant activity are similar between queen and worker ants | Parker et al., 2004 |
| | | Levels of antioxidant gene expression are similar between queen and worker honey bees | Corona et al., 2005 |
| | | Levels of antioxidant are lower in queen compared to worker honey bees | Hsieh and Hsu, 2013; Hsu and Hsieh, 2014 |

## 1.7 Aim and research questions

The overall aim of this thesis is to investigate the relationship between membrane phospholipid composition and longevity in different castes of honey bees (*Apis mellifera*). This thesis is divided into three main sections addressing the following aims:

Part A investigates the role of membrane polyunsaturated fatty acids (PUFA) in the longevity of honey bees. This section involves a comparison of the composition of membrane phospholipid molecules of the three bee castes: queens, workers, and drones at six different life-history stages from a single colony of free-living bees (Chapter 2). It also includes a comparison of membrane phospholipid molecules of adult workers at different ages covering the first 21 days of adult life, also from a free-living colony (Chapter 3).

Part B (Chapter 4) investigates an experimental test of the pacemaker theory of ageing to confirm if the shorter lifespan of workers is due to an increase in the propensity of membranes to peroxidise following pollen consumption.

Part C (Chapter 5) investigates different physiological parameters implicated in longevity i.e. metabolic rate as a generator of free radicals, and lipofuscin or AGE pigment as a glycation end-product of oxidative processes.

# PART A. Investigation of membrane phospholipids in the three castes of honey bees (*Apis mellifera*).

# Chapter 2. Honeybee caste lipidomics in relation to life-history stages and the long life of the queen.

# 2.1 Introduction

Social insects represent a promising model for the study of ageing. Ants, termites, and honey bees have all evolved a caste system with striking differences in lifespan between genetically identical long-lived queens and short-lived workers (Lucas and Keller, 2014). In honey bees, workers live for weeks whilst queens can live for years. This difference in lifespan readily surpasses any experimentally achieved lifespan extension (Keller and Jemielity, 2006).

Despite the vital role of honey bees in human food production and ecology, a very limited number of studies have looked at mechanism/s that could explain the extraordinary difference in lifespan between queens and workers. One study (Haddad et al., 2007) has suggested that distinctive differences in nutrition could explain differences in longevity between the female castes. A major difference in the types of food consumed by bees occurs after emergence as adult honey bees. Queens are fed mouth-to-mouth a fluid by worker bees (assumed to be the same royal jelly they receive as larvae). However, following emergence worker bees begin to consume honey and pollen in the form of 'bee bread' (a mixture of honey, pollen and glandular secretions) that is known to have a high polyunsaturated fatty acids (PUFA) content (Haddad et al., 2007; Manning and Harvey, 2002; Manning et al., 2007). For worker bees, this change in diet coincides with changes in their membrane phospholipids, with an increase in the proportion of PUFA and a decrease in the proportion of monounsaturated fatty acids (MUFA; Haddad et al., 2007). In queens, there is no change in the membrane fatty acid composition, which remains highly monounsaturated throughout life (Haddad et al., 2007; Robinson and Nation, 1970).

While the introduction of more than one double bond into a fatty acid chain affects the physical properties of the molecule, it can also affect its chemistry with substantial consequences. The double bonds of PUFA molecules are three carbons apart, and at least one section has the =CH-$CH_2$-CH= structure (see Figure 1.8 for details). Because a double bond weakens the bond energy of the C-H bonds on the next carbon atom, the H atoms attached to the middle C atom in this triplet (refer to as *bis*-allylic hydrogens) have the lowest bond energies in the total hydrocarbon chain and subsequently are most susceptible to removal by free radicals (Halliwell and Gutteridge, 2007). Only PUFA possess *bis*-

allylic C-H bonds, and when attacked by reactive oxygen species or other radicals, they produce carbon-centred radicals, which, in turn, initiate the autocatalytic process known as lipid peroxidation. The carbon-centred radical products of peroxidation of PUFA each, in turn, consume an oxygen molecule to produce a lipid peroxyl radical which can then attack another *bis-allylic* C-H bond on the same molecule or on a surrounding PUFA molecule to produce both, a lipid hydroperoxide as well as other carbon-centred radicals. In this way, an autocatalytic chain reaction is initiated (see Figure 1.9 for details). The lipid hydroperoxides can be further modified to produce a wide range of other reactive molecules that are responsible for oxidative damage to many other cellular components (Halliwell and Gutteridge, 2007).

Accumulation of oxidative damage throughout life forms the basis for the oxidative stress theory of ageing (reviewed in Beckman and Ames, 1998) originally proposed by Denham Harman as the free radical theory of ageing (Harman, 1956). A variation of this theory, the membrane pacemaker theory of ageing (Hulbert, 2005) emphasises the role of membrane lipid peroxidation and therefore PUFA in the free radical damage associated with aging. Several species of mammals, birds and some invertebrates show a strong relationship between the susceptibility of membranes to peroxidise and maximum lifespan (Hulbert et al. 2017). It is also of interest that this correlation is found between highly variable species within animal classes, within similar species or even variants of the same species with different longevities (Hulbert et al., 2006a, 2014, 2017) as well as in calorie restriction (Faulks et al., 2006). The common finding of an association between the membranes susceptibility to peroxidise and maximum lifespan in invertebrates and vertebrates supports the implication of PUFA peroxidation as a central mechanism in the biology of aging.

To investigate the role of membrane phospholipid PUFA in the longevity of honey bees, the current study compares the molecular phospholipid composition of three bee castes; queens, workers, and drones at six different life-history stages from a single colony of free-living bees.

## 2.2 Material and Methods

### 2.2.1 Source of Honey Bees

Different life history stages of three honey bee castes were sampled from the same location in Grenfell, New South Wales (NSW), Australia (GPS-33.901249 S, 148.173194 E). All bees were immediately frozen and stored in dry-ice during transportation and then upon arrival at the University of Wollongong, NSW, Australia were subsequently stored at -80°C.

### 2.2.2 Caste sampling

Drones and workers of various ages were sampled from one hive during early austral summer (December 2014). Queen bees were manually grafted using eggs laid by the queen that produced the same workers and drones used in this study. Briefly, a small larva from a worker cell was transferred to a plastic cell and then moved into a queen-less hive. The plastic queen cells were monitored for 10 days before transfer into their new hive (at that stage, queen cells were sealed). Queens larvae were sampled at day 3 (categorize as early larva, $n = 3$) and day 6 (categorize as late larva, $n = 5$). After 10 days, some of the queen cells were transferred into an incubator maintained at 35°C with a relative humidity of 70 ± 5%. Pupa queen (day 12, $n = 5$) and emergent queens were also sampled after hatching from their respective cells inside the incubator. The remaining sealed queen cells were then transferred into new queen-less hives. As each queen emerged, it was tagged 5-7 days post-emergence (see tagging methods in section Chapter 3.2.1) and monitored in natural conditions during the next 12 months. Six (6) queens were randomly sampled for lipid characterisation in December 2015 (categorize as young queens, 12-month-old). Previously marked queens of 3 years of age were sampled in austral summer of 2014 ($n = 4$). This experimental design reduced any genetic differences between the individual samples.

The different life history stages of worker bees and drones (male caste) was determined by a professional apiarist, Greg Brenner (20 years of experience in the apiary industry). Each bee was selected according to the criteria listed in Table 2.1. Pollen (as "bee-bread") was collected from the same hive for fatty acids analysis. Total fatty acids composition of pollen (Table 2.2) was performed as previously described using gas chromatography (Abbott et al., 2010).

Table 2. 1 Criteria used to sample honey bees (*Apis mellifera*).

| Stages | Description |
|---|---|
| Early larva | small size larva |
| Late larva | large size larva |
| Pupa | cell capped, bee with soft exoskeleton, hairless, eyes developed, no wing development |
| Emergent | collected after emerging from the capped cell, still with soft exosquelette, no contact with another bee, haven't flown as yet |
| Nurse (workers) | hair fully developed, collected in the hive doing nursing related tasks, without pollen on their legs |
| Forager (workers) | collected near the entrance of the hive, carried pollen, wings still intact; bright color |
| Young queens (12 months old) | Marked after emerging and sampled at 12 months |
| Old queens (3 years old) | Marked after emerging and sampled at three years |
| Young drones | Hair fully developed and collected in the hive, wing still intact, bright colour, large eyes |
| Old drones | Collected inside the hive, wings wearing-out, dark colour, large eyes |

Larvae and pupae of workers were collected from regular sized honeycomb cells. Larvae and pupae of drones were collected from larger honeycomb cells. Larvae and pupae of queens were collected from artificial cells used to produce queens.

Table 2. 2– Relative proportion of total fatty acid composition of "bee bread" pollen.

| Fatty acids | % of total fatty acids |
|---|---|
| 14:0 | 4.2 ± 0.4 |
| 16:0 | 21.5 ±0.9 |
| 16:1 | 0.9 ± 0.7 |
| 17:0 | 6.8 ± 0.6 |
| 18:0 | 2.9 ± 0.3 |
| 18:1 | 5.2 ± 2.1 |
| 18:2 | 28.9 ± 1.2 |
| 18:3 | 28.7 ± 1.0 |
| 20:0 | Trace |
| 20:1 | 0.8 ± 0.6 |
| Total SFA | 35.5 ± 1.5 |
| Total MUFA | 6.9 ± 2.9 |
| Total PUFA | 57.7 ± 1.4 |
| Peroxidation index | 86.4 ± 2.2 |

Total lipid fatty acids, *n* =3. Data are expressed in mol % and as mean ± s.e.m.

### 2.2.3 Molecular phospholipids

Analysis of molecular phospholipids was performed as previously described using mass spectrometry (Cortie et al., 2015; Mitchell et al., 2007). Phospholipid species were extracted from bees (head + thorax + abdomen) as a previous study had shown that membrane fatty acid composition of the three body segments were very similar (Haddad et al., 2007). However, the stinger and attached venom sac of each bee were carefully removed due to the presence of phospholipase A2 in bee venom. Extracts without removal of the stinger and venom sac showed a significant increase in lysophospholipids (data not show).

Bee body mass was measured followed by leg removal to avoid contamination by pollen that is known to have a high PUFA content. Each bee was homogenized in a Geneworks® homogenization vial filled with ceramic beads using two passages of 60 sec at 6 m/s in 10 volumes of bulk solvent mix (MeOH + internal standard) repeated with MeOH only during a second homogenisation to remove all remnants of the bee. For each phospholipid class, an internal standard with acyl chains not detected in honey bees was added to allow for quantification of each phospholipid class. Butylated hydroxytoluene (0.01% w/v) was added to all solvents as an antioxidant to preserve fatty acid composition. To extract lipids, each bee homogenate was vortexed (Mix mate, Thermofisher, Scoreby, VIC, Australia) for an hour at 4°C in 2 mLs of methyl-tert butyl ether (MTBE), 300µL of 150 mM of ammonium acetate (AmOAc) was added and vortexed for a further 15 min at 4°C (Matyash et al., 2008). Following this, the homogenate was centrifuged at 20,000*g* for 5 min and the lipid-containg MTBE (top) phase was removed and stored under nitrogen gas at -20°C.

Nano-electrospray ionization mass spectrometry was performed on lipid extracts using a hybrid triple quadrupole linear ion trap mass spectrometer (QTRAP® 5500 AB Sciex, Framingham, MA, USA) equipped with an automated chip-based nano-electrospray source (TriVersa Nanomate$^{TM}$, Advion Inc., Ithaca, NY, USA). On the day of analysis, samples were diluted with MeOH: $CHCl_3$ (2:1) containing 5 mM AmOAc to an optimal concentration of approximately 10 µM of total phospholipids. Samples were loaded onto 96-well plates, centrifuged (10 min, 2200*g*) and directly infused into the mass spectrometer. Spray parameters were optimized at a gas pressure of 0.4 psi with a voltage of 1.2 kV and 1.1 kV for positive and negative ion modes respectively.

Glycerophospholipid MS/MS prediction tool (www.lipidmaps.org) was used to make target lists and converted to targeted ion lists used in Lipidview™ (version 1.3, AB Sciex, Framingham MA, USA). Lipidview™ software was set at a mass tolerance of 1 DA, with a minimum intensity of 1 % and a minimum signal-to-noise ratio of 10. Positive precursor ion scans were used to quantify phospholipid molecules for lysophosphatidylcholine (LPC), phosphatidylcholine (PC), lysophosphatidylethanolamine (LPE), phosphatidylethanolamine (PE) and phosphatidylserine (PS). A negative precursor ion scan was used to quantify phospholipid molecules for phosphatidylinositol (PIn). Negative precursor ion scans were used to identify fatty acids using a custom-made spreadsheet in Microsoft Excel 2014 (Microsoft Corporation, Redmond WA, USA). A list of all precursor ion scans used is provided in appendix 1. Phospholipids were quantified at the sum composition level (e.g., PC 36:2) for each respective phospholipid head group before the molecular phospholipid level was determined from fatty acid scans (e.g., PC 36:2 can be PC 18:0_18:2 or PC 18:1_18:1).

The sn-1 and sn-2 positions of each fatty acid on the molecular phospholipid species could not be identified using the current method and isomeric phospholipid species containing alkyl ethers (termed O=) or vinyl ethers (plasmalogens, termed P=) could not be differentiated. Those two isomeric molecules were interpreted as plasmalogen in the current study. Individual phospholipid molecules were quantified by comparison with internal phospholipid standards (see Appendix 2) of the same class after correction for isotope contribution. Phospholipid molecules are reported as nmol of molecular phospholipid per mg of bee (nmol· $mg^{-1}$). Phospholipid structure is reported using the nomenclature described by Liebisch et al., (Liebisch et al., 2013). The list of all individual molecular phospholipids compiled in the three castes of honey bees is presented in Appendix 3.

### 2.2.4 Fatty acid composition

Membrane fatty acid composition as percent of total fatty acid was calculated via the quantification of fatty acids from the phospholipid molecules present in the extract using a formulated Excel spreadsheet (Microsoft Corporation, WA, USA). Total fatty acids combined all molecular phospholipids quantified by Lipidview™. Fatty acids are expressed as mol % of total fatty acids as well as nmol. $mg^{-1}$of bee.

### 2.2.5 Peroxidation index

The membrane peroxidation index of whole bee lipid extracts was calculated from the percent of total membrane phospholipid fatty acid compositions as the sum of *bisallylic* bonds per 100 fatty acids according to the equation:

Peroxidation Index PI = (∑ % di-PUFA *1) + (∑ % tri-PUFA*2) + (∑ % tetra-PUFA*3) + (∑ % penta-PUFA*4) + + (∑ % hexa-PUFA*5).

### 2.2.5 Statistical analysis

Membrane phospholipids were compared between the castes and life-history stage using an Analysis of variance (two-way ANOVA) with a Tukey post hoc, or non-parametric Kruskal-Wallis tests, with a Wilcoxon post hoc, corrected by Bonferroni. All analyses were performed with R software (Version 3.2.2).

## 2.3 Results

### 2.3.1 Body mass

During development from larvae to older adults, body mass increased from a few milligrams to between 100-296 mg in the different castes (Table 2.3). In worker bees, body mass increased after emergence reaching a maximum body mass of ~160 mg in young workers (i.e. nurses) before reducing in the older foragers to be approximately the same body mass as emergent workers (~100mg). Emergent queens had larger body masses than emergent workers (43% increase) reaching a maximum body mass (as measured) of 284 mg at 12 months. Emergent drones had the largest body masses of all the castes (61% increase compared to workers and a 32% increase compared to queens) with their body mass then reducing (~22%) as adults.

### 2.3.2 Fatty acids

The fatty acid composition of membrane phospholipids of three castes at six different life stages from a free-living colony of bees was analyses both as relative percentage (mol %; Figures 2.1) and as changes in total amount (nmol. $mg^{-1}$of bee; Figures 2.2).

Table 2. 3. Body mass (mg) of different bee castes

| Life stage | *n* | Workers | *n* | Queens | *n* | Drones |
|---|---|---|---|---|---|---|
| Early larva | 10 | 1.6 ± 0.2[a] | 3 | 16.4 ± 6.1[b] | 9 | 21.9 ± 1.7[b] |
| Late larva | 10 | 76.5 ± 4.9[a] | 5 | 207 ± 36[b] | 10 | 85.7 ± 13[a] |
| Pupa | 10 | 106 ± 5.3[a] | 5 | 255 ± 5.2[b] | 7 | 296 ± 17[b] |
| Emergent | 9 | 105 ± 1.0[a] | 5 | 183 ± 7.3[b] | 5 | 269 ± 6.2[c] |
| Young adult | 10 | 159 ± 5.7[a] | 6 | 284 ± 6.7[b] | 8 | 211 ± 6.7[c] |
| Old adult | 10 | 100 ± 4.0[a] | 4 | 255 ± 6.8[b] | 10 | 208 ± 1.8[c] |

Young adult queens were 12-month-old. Old adult queens were three-years-old. Data are presented as mean ± SEM and expressed in milligram (mg). Letters (a, b, c) indicate a difference between the castes for the same life-history stage (e.g. body mass is similar between early larva queens and early larva drones but significantly higher compared to early larva workers. Level of significance was set at $p < 0.05$ when comparing castes (e.g. early larva workers versus early larva queens versus early larva drones).

### 2.3.3 Relative saturated fatty acids (SFA)

The relative proportion of SFA (Figure 2.1-A) in membrane phospholipids of all three different castes showed a similar trend during development with a decrease in relative levels leading to ~25% at emergence. However, following emergence workers and drones further reduced the proportion of SFA in their membranes whereas adult queens maintained relatively stable SFA levels (similar to earlier development). Queens maintained a significantly higher proportion of SFA in their membranes during adult life compared to workers and drones ($p < 0.05$; Figure 2.1-A).

### 2.3.4 Relative monounsaturated fatty acids (MUFA)

At various stages in the life-history of bees (notably in queens and drones), MUFA were the dominant membrane fatty acids accounting for as much as 80% of total fatty acids (Figure 2.1-B). The relative level of MUFA in membranes decreased continuously throughout development in workers whereas in drones the decrease was limited to the early developmental period up to pupa (Figure 2.1-B). Whilst queens maintained significantly higher levels of MUFA compared to other castes at pupation and emergence ($p < 0.001$, Figure 2.1-B). Following emergence, queens reduced their level of total MUFA while drones increased them. As old adults, drones had a significantly higher level of MUFA compared to the female castes, and queens had a significantly higher level of MUFA compared to workers (Figure 2.1-B).

### 2.3.5 Relative polyunsaturated fatty acids (PUFA)

All castes maintained a very low proportion of PUFA during their larval stages (~2%, Figure 2.1-C). During this early developmental period, the most notable difference was a consistent increase in membrane PUFA in workers and drones compared to queens. After emergence as adults, workers continued to increase their level of PUFA reaching a maximum level of ~15% of membrane fatty acids as young adults. Queens also increased the level of membrane PUFA in their membranes after emergence but to a lesser extent (~7.5%) compared to workers. In contrast, drones reduced their level of membrane PUFA during adult life (~4%).

### 2.3.6 Peroxidation index (PI)

Membrane peroxidation index (PI) was maintained at similarly low levels during the larval stages in all castes at ~2.5 (Figure 2.1-D). However, leading to emergence both workers and drones increased their PI values up to ~12 by incorporating PUFA into their membrane phospholipids (Figure 2.1-D). In contrast, queens maintained consistently low peroxidation index values throughout this period (Figure 2.1-D). At emergence as adults, membrane PI was 2-fold higher in workers and drones compared to queens (Figure 2.1-D, $p < 0.001$). The largest increase in PI was observed in workers after they emerged with membrane PI's increasing by 2.5-fold in young adults and remaining high thereafter (value of ~27). Overall, there was a 10-fold increase in peroxidation index of workers from early larva to old adults. Queens also increased their membrane PI following emergence but to a much lesser extent. Interestingly, membrane PI of three years old adult queens was similar to that of emergent workers (~10). In drones, PI reduced during adult life with old drones having similar membrane PI values to old queens (Figure 2.1-D).

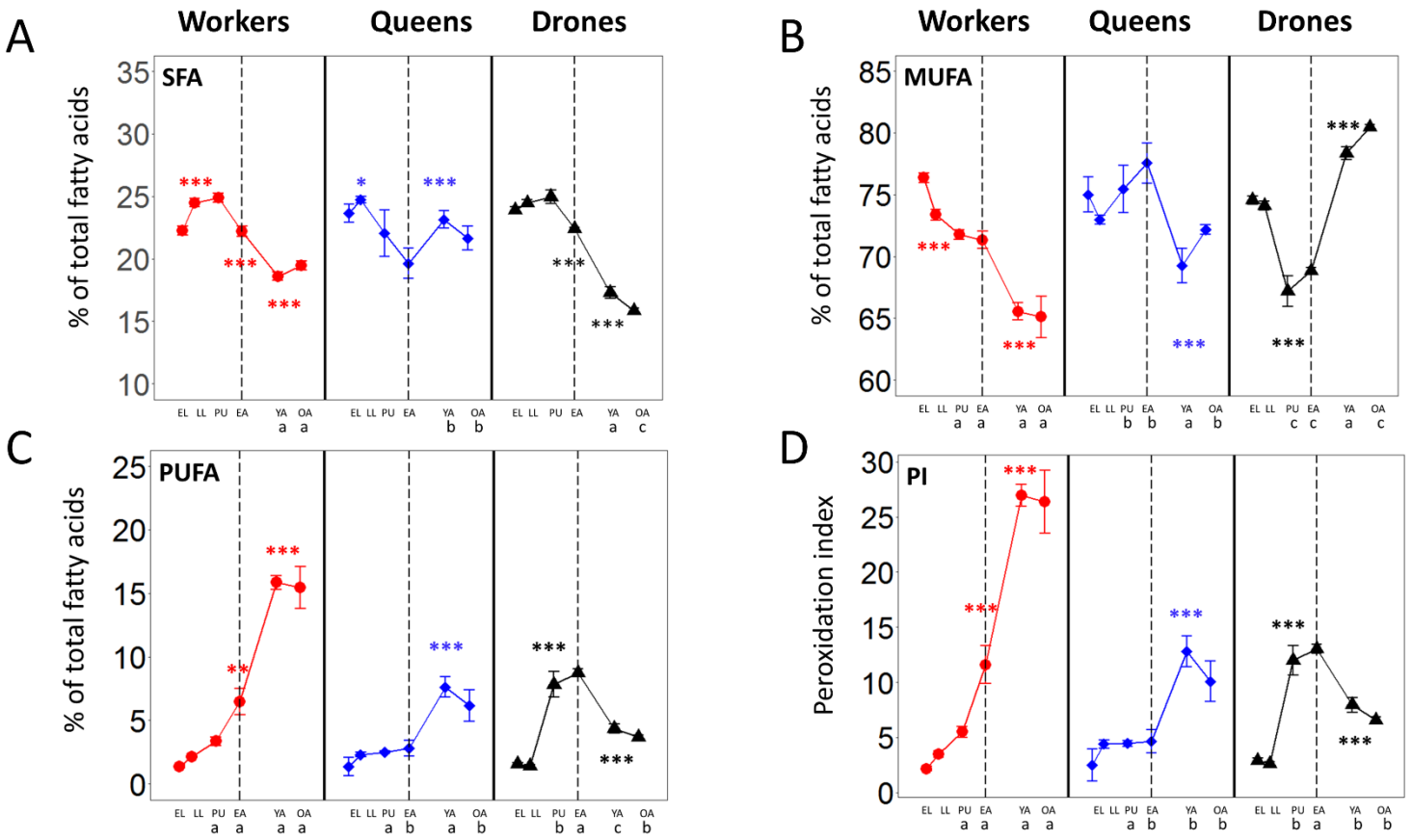


Figure 2.1**.** Relative proportion of saturated fatty acids (SFA; **A**), monounsaturated (MUFA; **B**) and polyunsaturated (PUFA; **C**) in membrane phospholipid extracts of the three different castes of honey bees (*Apis mellifera*). Peroxidation index (PI) of membrane phospholipid extract is presented in **D**. Each caste has six life-history stages described as (from left to right): EL: early larva, LL: late larva, PU: pupa, EA: emergent adult, YA young adult and OA: old adults (see Table 2.11 for further details on life-history stage description and Table 2.1 for sample size). Dash line indicate emergence as adult. Data are presented as mean ± s.e.m. and expressed as percent of total fatty acids (mol %). Letter on x axis indicates significant difference among the castes for a given life-history stage with $p < 0.05$ (e.g. in **A**, Young adult worker and drones differ significantly to young adult queens). Asterisk indicates significant change from previous life-history stages within the same caste (i.e. from early larva to late larva, in workers) with * $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$.

### 2.3.7 Total fatty acids

The total fatty acids (expressed as nmol of fatty acids· $mg^{-1}$ of bee) showed a similar trend among the castes. All castes decreased their level of total fatty acids during development up to pupation. After pupation, total fatty acids increased following emergence and development as young adults by up to 65%, in queens and drones (Figure 2.2-A). The increase in total fatty acids was primarily driven by large post-emergence increases in total MUFA levels (Figure 2.2-C) in both castes and to a lesser extent, an increase in total SFA (Figure 2.2-B) and total PUFA (Figure 2.2-D) in queens. The increase in total fatty acids presumably represented the building of new membranes during early adulthood in drones and queens. The increase in total fatty acids appears to be delayed in workers as young adults had similar levels of total fatty acids compared to emergent workers. The change in old adult worker membranes was also driven mainly by an increase in total MUFA (Figure 2.2-C) but also an increase in total PUFA (Figure 2.2-D) and to a less extent total SFA (Figure 2.2-B). Regarding total fatty acids changes, drones changed very little in either SFA or PUFA (Figure 2.2-B and 2.2-D) following emergence but increased MUFA by 2-fold (Figure 2.2-C). Presumably the delayed increase in total SFA and MUFA, and increase in PUFA in older workers (i.e. foragers) is due to the increased presence of membranes from young adult stage (nursing) to old adult stage (foraging), whereas queens and drones appear to synthesise membranes immediately following emergence (as indicated by Figures 2.2-A). The production of eggs in adult queens may also contribute to higher membrane content in young adult stage.

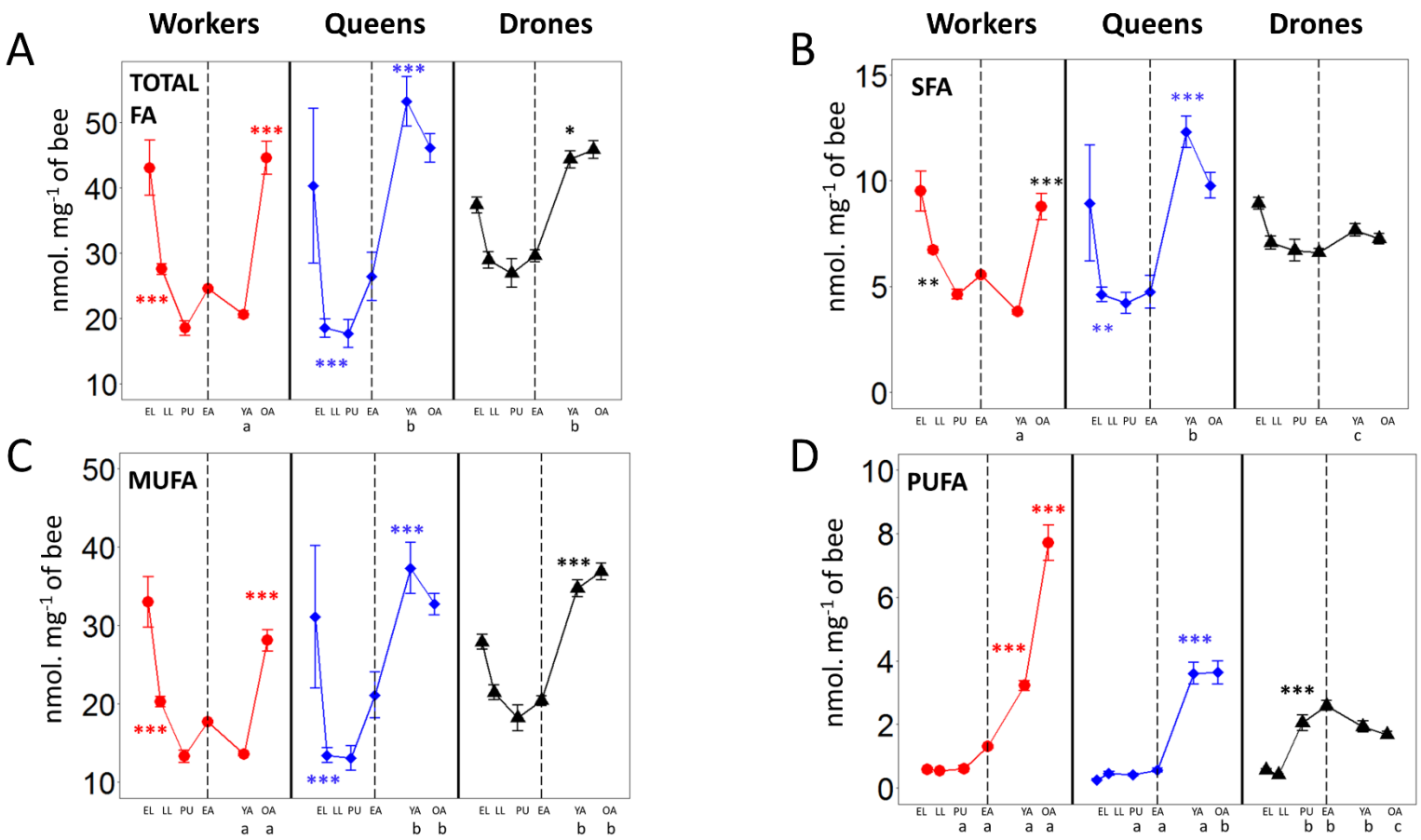


Figure 2.2. Total fatty acids of membrane phospholipid extracts of the three different castes of honey bees (*Apis mellifera*). Fatty acids are presented as total fatty acids (Total FA; **A**), total saturated fatty acids (SFA; **B**), total monounsaturated fatty acids (MUFA: **C**) and total polyunsaturated fatty acids (PUFA; **D**). Each caste has six life-history stages characterised. Stages are described as (from left to right): early larva (EL), late larva (LL), pupa (PU), emergent adult (EA), young adult (YA) and old adult (OA) bees. Data are presented as mean ± s.e.m. and expressed as nmol. $mg^{-1}$of bee. Letter on x axis indicates significant difference among the castes for the a given life-history stage with $p < 0.05$. Asterisks indicate a significant change from previous life-history stage within the same caste with * $p$ <0.05, ** $p < 0.01$, ***$p < 0.001$.

Since only phospholipids containing PUFA contribute to membrane peroxidation index (PI), the main contributors to PI (85%) in adult worker bees were from phosphatidylcholine (PC) and phosphatidylethanolamine (PE) phospholipids that contained PUFA (see Figure 2.3). In adult queens, more than 50% of membrane PI was derived from phosphatidylinositol (PIn) molecules that contained PUFA. In drones, from early development to emergence ~50% of PI was also derived from phosphatidylinositol molecules containing PUFA but once drones became adults most of the PUFA contribution to PI reverted to PUFA from phosphatidylcholine and phosphatidylethanolamine molecules, as found in workers.

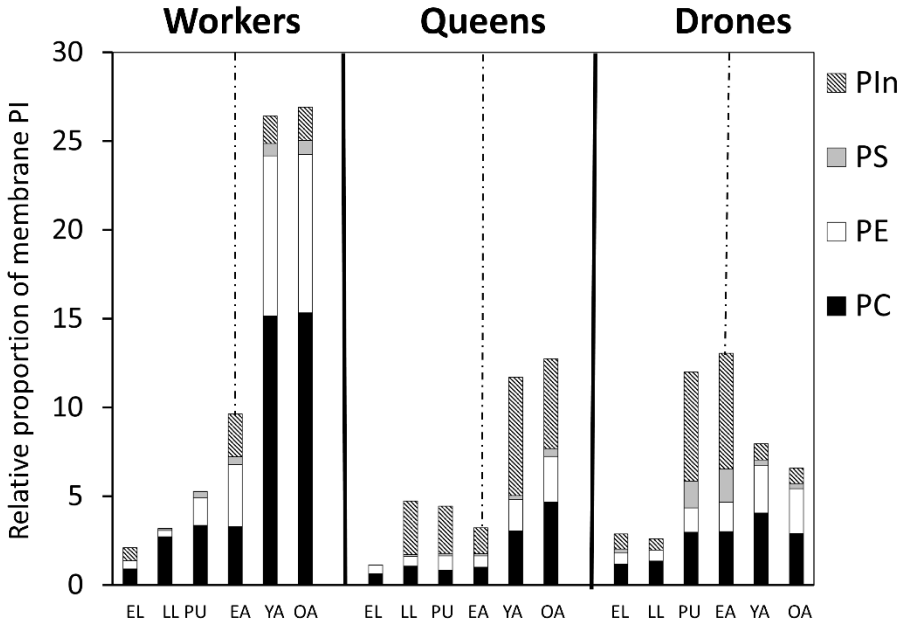


Figure 2.3 Contribution of the different phospholipid headgroups to peroxidation index of membrane phospholipid extracts of the three different castes of honey bees (*Apis mellifera*). Each caste has six life-history stages characterised. Stages are described as (from left to right): early larva (EL), late larva (LL), pupa (PU), emergent adult (EA), young adult (YA) and old adult bees (OA). Data are presented as proportion of each headgroup of total peroxidation index.

### 2.3.8 Total phospholipids

The three castes showed similar changes in their membrane densities over development as indicated by total phospholipid (in nmol. $mg^{-1}$of bee; Figure 2.4) in a U-

shaped curve indicating decreases in membrane density during early development, followed by increases in adult life. Interestingly, levels of total phospholipids in old adults were similar, and at about the same level as they were in the early larval stage.

When comparing the three castes at similar life-history stages, workers and drones had a higher content of total phospholipid compared to queens in the late-larva stage ($p < 0.001$) whereas the pupa of workers and queens had similarly lower contents of total phospholipid compared to drones ($p < 0.01$). As young adults, workers had a lower content of total phospholipid compared to queens and drones ($p < 0.001$) whereas all castes had a similar amount of total phospholipids as old adults (~ 22 nmol. $mg^{-1}$of bee).

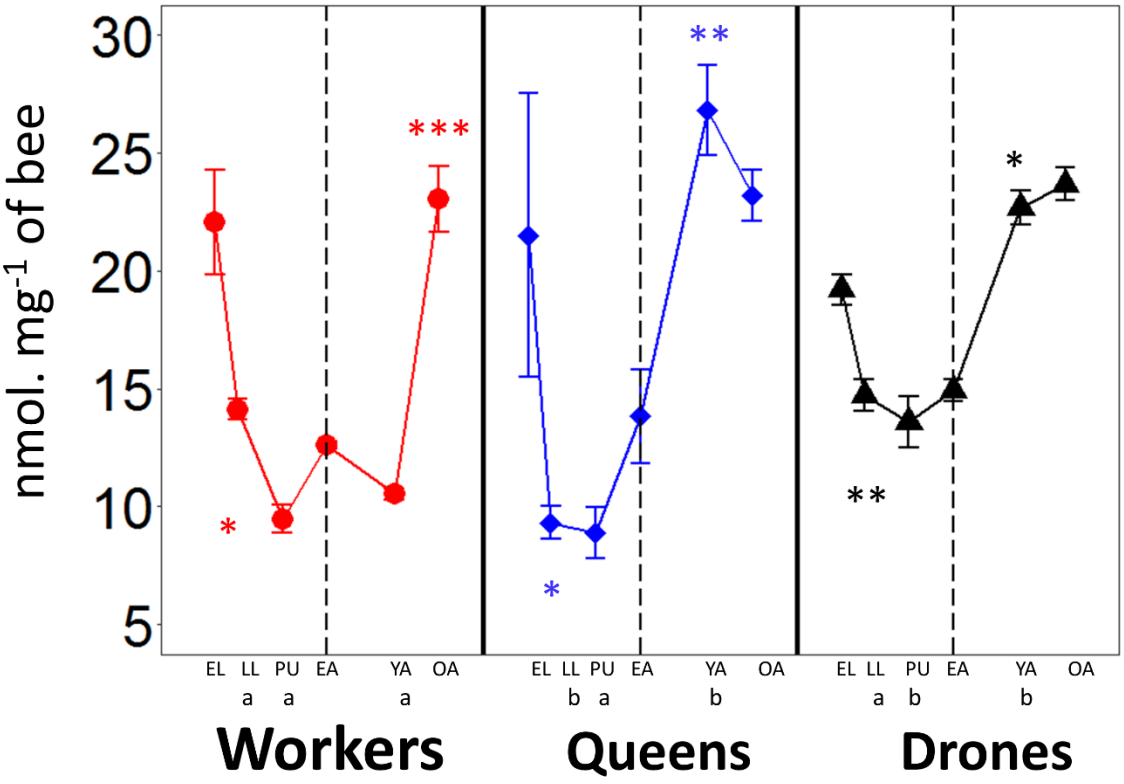


Figure 2. 4. Amount of total phospholipid in lipid extracts of the three different castes of honey bees (*Apis mellifera*). Each caste has six life-history stages characterised. Stages are described as (from left to right): early larva (EL), late larva (LL), pupa (PU), emergent adult (EA), young adult (YA) and old adult (OA) bees. Data are presented as mean ± s.e.m. and expressed as nmol. $mg^{-1}$ of bee. Letters indicate a significant difference among the castes for a given life-history stage with $p < 0.05$. Asterisks indicate a significant change from previous life-history stage within the same caste with * $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$.

### 2.3.9 Phospholipid classes

A quantitative comparison of the four major classes of membrane phospholipids from lipid extracts of whole bees of different castes and life stages of bees is presented in Figures 2.5. Phosphatidylcholine (PC; Figure 2.5-A) and phosphatidylethanolamine (PE; Figure 2.5-B) were the main phospholipid classes found in membranes with phosphatidylinositol (PIn; Figure 2.5-C) and phosphatidylserine (PS: Figure 2.5-D) being of lower abundance. In general, the castes showed similar patterns of change during their development. It was commonly observed in all three castes that the level of PC, PE and PS in older adults was very similar to that found in early larva. Despite numerous statistical differences, most differences between the castes remained fairly small, except PIn that expressed much higher levels in queens as adults (Figure 2.5-C). It was also observed that PS appeared relatively constant in drones throughout their development whereas both female castes showed 2-3 fold changes at different stages of their development (Figure 2.5-D).

A general observation for PC was that all castes underwent similar changes during development (see Figure 2.5-A). The general trend observed for PC molecules was a reduction during larval development followed by an increase after emergence as adults. The only notable difference for PC was in young workers, when PC remained low (reflected in total phospholipids). Drones were also found to possess less variable levels of PC during their development compared to female castes (Figure 2.5-A).

Changes in PE levels during development were similar to those seen for total phospholipids and PC with a U shaped curve from larva to old adult (Figure 2.5-B). As occurred for PC, the profile of PE showed a delayed increase in young adult workers (remaining similar to levels at emergence), a change that was not present in young queens or drones (Figure 2.5-B).

Unlike other phospholipid classes, PIn demonstrated a large amount of variability between the castes. Queens were found to significantly increase their PIn levels throughout life from early larva to young adult stages (from 0.5 to 3 $nmol \cdot mg^{-1}$, see Figure 2.5-C). Drones showed a similar increase as queens up to emergence but then reduced their level of PIn in adulthood. Workers showed low levels of PIn and little change throughout development.

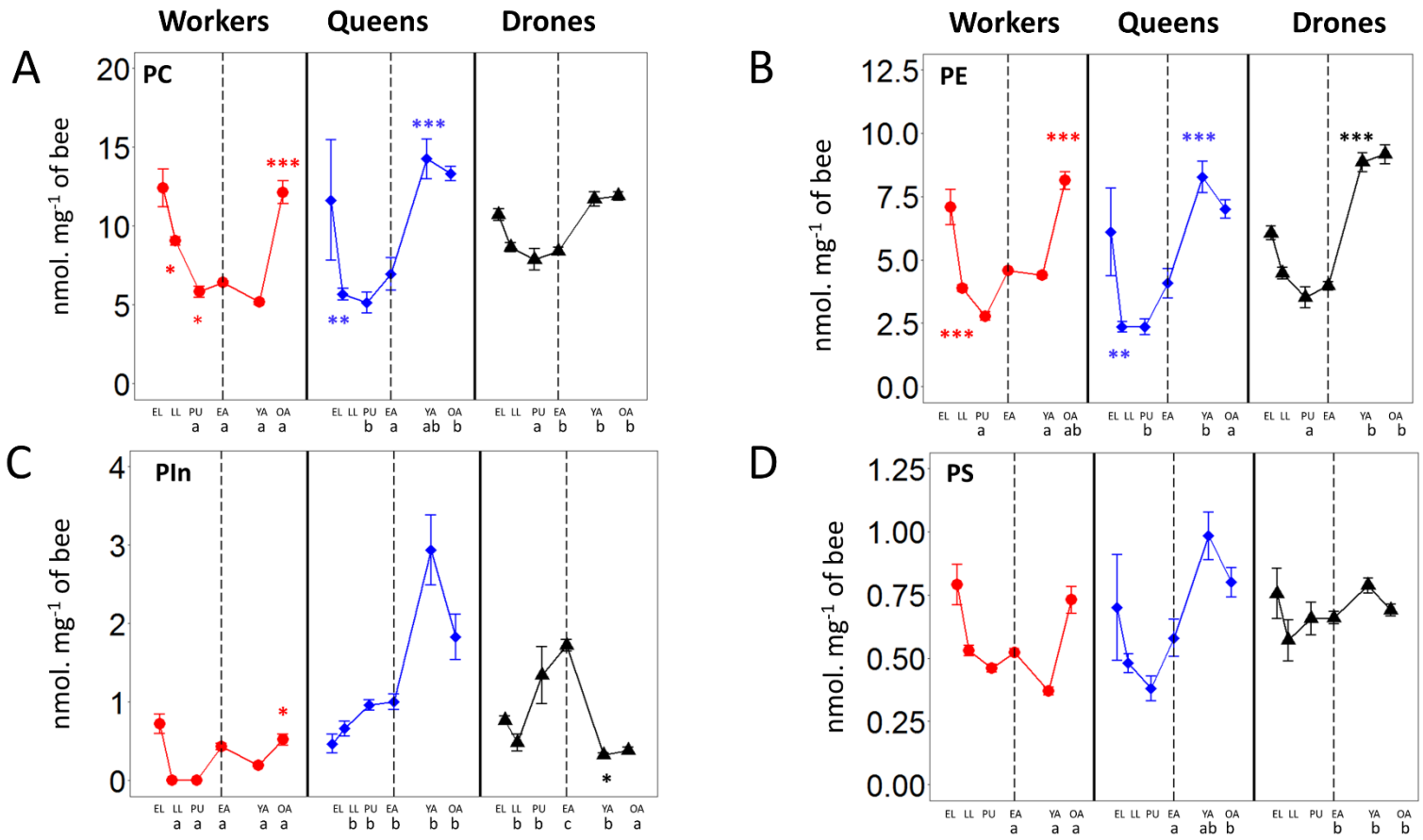


Figure 2. 5 - Amount of the main phospholipid classes in lipid extract of the three different castes of honey bees (*Apis mellifera*). Phospholipid classes are **A**: Phosphatidylcholine (PC); **B**: phosphatidylethanolamine (PE); **C**: phosphatidylinositol (PIn) and **D**: phosphatidylserine (PS). Each caste has six life-history stages characterised. Stages are described as (from left to right): early larva (EL), late larva (LL), pupa (PU), emergent adult (EA), young adult (YA) and old adult (OA) bees. Data are presented as mean ± s.e.m. and expressed as nmol · $mg^{-1}$ of bee. Letter on x axis indicates significant difference among the castes for the a given life-history stage with $p < 0.05$. Asterisk indicates significant change from previous life-history stages within the same caste with * $p < 0.05$ ,** $p < 0.01$, ***$p < 0.001$.

Despite a few significant differences among the castes, the levels of PS were quite low compared to other phospholipids (level < 1 nmol. $mg^{-1}$; see Figure 2.5-D). Female castes showed a reduction in their total level of PS from early larva to pupa. As previously found for both PC and PE, young adult workers had an initial hiatus in PS increases waiting till older adulthood to double their PS levels whereas queens continued to increase their PS levels from pupae to young adult (reaching the highest level of PS at ~1 nmol. $mg^{-1}$). In contrast to the female castes, drones maintained a relatively constant level of PS throughout development (Figure 2.5-D).

### 2.3.10 Lysophospholipids

There were few statistical differences in total lysophospholipids between castes (Figure 2.6). One notable difference was the increase in lysophospholipids in older adults in both workers and drones compared to queens ($p < 0.01$), with queens maintaining low levels of lysophospholipids in adulthood (Figure 2.6). Workers and drones had a higher level of total lysophospholipids compared to queens at the late larval stage (Figure 2.6, $p < 0.01$). When emerging as adults, queens had a slightly higher level of total lysophospholipids compared to drones ($p < 0.05$). In young adults, the level of total lysophospholipids was higher in drones compared to workers and queens ($p < 0.01$). Overall, the total concentrations of lysophospholipids were an order of magnitude lower than those of total phospholipids.

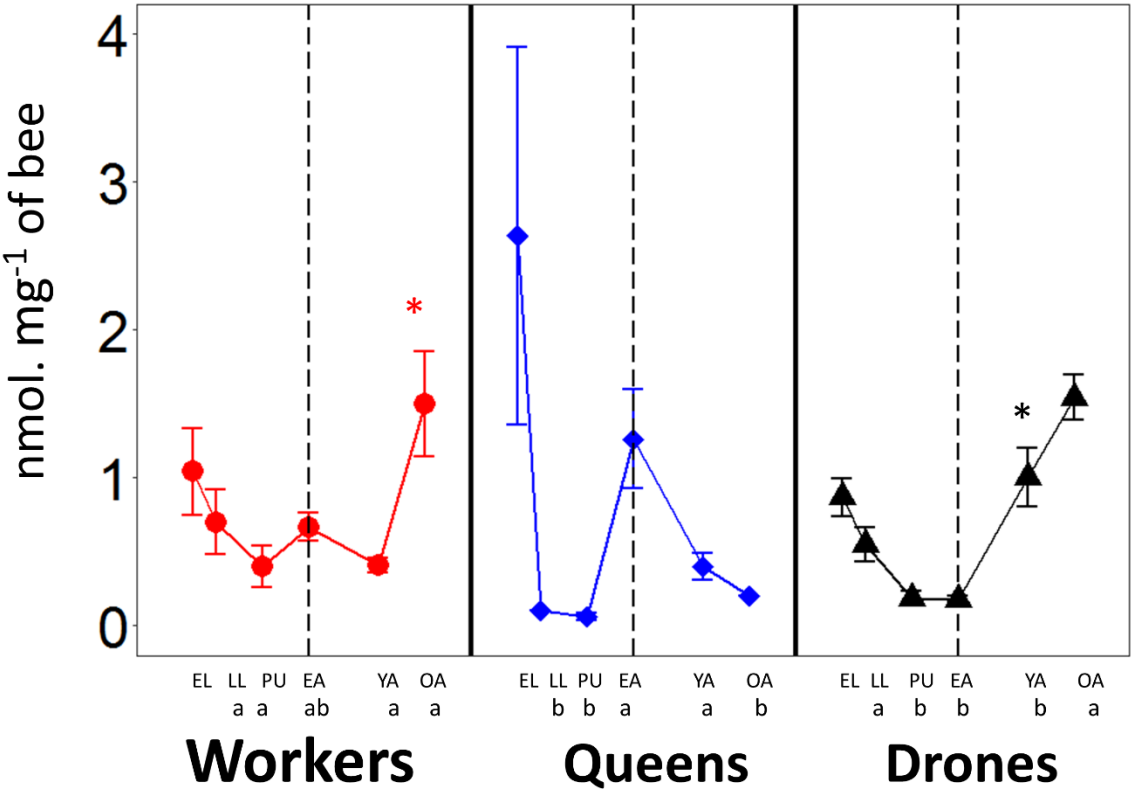


Figure 2. 6. Amount of lysophospholipids in lipid extracts of the three different castes of honey bees (*Apis mellifera*). Each caste has six life-history stages characterised. Stages are described as (from left to right): early larva (EL), late larva (LL), pupa (PU), emergent adult (EA), young adult (YA) and old adult (OA) bees. Data are presented as mean ± s.e.m. and expressed as nmol. $mg^{-1}$of bee. Letters indicate a significant difference among the castes for a given life-history stage with $p < 0.05$. Asterisks indicate a significant change from previous life-history stage within the same caste with * $p < 0.05$.

### 2.3.11 Major phospholipid molecules

#### *2.3.11.1 Non-PUFA contains molecular phospholipids*

Tables 2.4 and 2.5 shows the main phospholipids that contained saturated and monounsaturated fatty acids (i.e. 16:0_18:1; 18:0_18:1; 16:1_18:1 and 18:1_18:1) in the four most abundant phospholipid classes. A common trend observed in abundant non-PUFA-containing phospholipid species for PC, PE and PS was a decrease from early-larva to pupa stage followed by an increase in adult life (i.e. similar change as total phospholipids). The most abundant non-PUFA-containing phospholipid molecule in all castes was PC 18:1_18:1 followed by PC 16:0_18:1. For PE phospholipids the most abundant non-PUFA-containing

combinations were PE 18:1_18:1 and PE 18:0_18:1. For PIn, non-PUFA-containing phospholipids were in extremely low abundance in workers whereas in queens they were in relatively high abundance and, in drones in moderate abundance, especially during adult life. In queens, the PIn molecule with the greatest abundance was PIn 18:0_18:1. In PS, the most common non-PUFA-containing molecule was almost equally shared by PS 16:1_18:1 and PS 18:1_18:1 in all castes. In general PS phospholipids demonstrated more limited differences between the different castes.

The non-PUFA-containing phospholipid molecules within PC, PE, and PS, displayed very similar levels in the different castes up to emergence. However, as young adults, workers were consistently lower (2 to 5-fold) compared to queens and drones. In old adults, these differences became diminished between the castes. Another interesting observation was the difference in PIn levels among the different castes. Queens, particularly as adults, had a much higher level of PIn 18:0_18:1 compared to adult workers and adult drones. Queens and drones also had a higher level of PIn 18:1_18:1 compared to workers during development. Overall, despite numerous statistical significant differences, the castes had fairly similar levels of most of non-PUFA-containing molecular phospholipids, except PIn 18:0_18:1 that was much higher in queens compared to workers and drones.

Table 2. 4 – Main molecular phospholipids characterised in phosphatidylcholine (PC) and phosphatidylethanolamine (PE) of bee extract. Phospholipids in this table contain only saturated (SFA) and monounsaturated fatty acid (MUFA).

| | | Workers | Queens | Drones | | | Workers | Queens | Drones |
|---|---|---|---|---|---|---|---|---|---|
| PC 16:0_18:1 | Early larva | 3.6 ± 0.4 | 3.9 ± 1.4 | 3.5 ± 0.2 | PE 16:0_18:1 | Early larva | 1.1± 0.1*** | 1.0 ± 0.4 | 0.9 ± 0.03 |
| | Late larva | 3.5 ± 0.1$^{a}$ | 1.8 ± 0.1$^{b}$ | 2.8 ± 0.1$^{c}$*** | | Late larva | 0.6 ± 0.02* | 0.4 ± 0.02*** | 0.6 ± 0.03*** |
| | Pupa | 1.8 ± 0.1$^{a}$*** | 1.5 ± 0.2$^{ab}$ | 2.3 ± 0.2$^{b}$ | | Pupa | 0.3 ± 0.01 | 0.3 ± 0.04 | 0.4 ± 0.04 |
| | Emergent adult | 1.5 ± 0.1$^{a}$ | 1.6 ± 0.6$^{ab}$ | 2.1 ± 0.1$^{b}$ | | Emergent adult | 0.3 ± 0.01 | 0.3 ± 0.06 | 0.3 ± 0.01 |
| | Young adult | 0.6 ± 0.02$^{a}$*** | 3.1 ± 0.2$^{b}$ | 2.1 ± 0.2$^{b}$ | | Young adult | 0.1 ± 0.04$^{a}$ | 0.8 ± 0.06$^{b}$*** | 0.4 ± 0.03$^{c}$ |
| | Old adult | 1.5 ± 0.1$^{a}$* | 2.3 ± 0.1$^{b}$ | 1.9 ± 0.1$^{b}$ | | Old adult | 0.30 ± 0.03 | 0.5 ± 0.04 | 0.4 ± 0.02 |
| PC 18:0_18:1 | Early larva | 0.5 ± 0.05 | 0.5 ± 0.1 | 0.5 ± 0.03 | PE 18:0_18:1 | Early larva | 1.9 ± 0.2 | 1.5 ± 0.4 | 1.6 ± 0.1 |
| | Late larva | 0.5 ± 0.02$^{a}$ | 0.3 ± 0.1$^{b}$ | 0.7 ± 0.04$^{c}$ | | Late larva | 1.2 ± 0.1*** | 0.8± 0.1 | 1.4 ± 0.1 |
| | Pupa | 0.4 ± 0.02 | 0.3 ± 0.04 | 0.5 ± 0.07 | | Pupa | 1.0 ± 0.04 | 0.8 ± 0.1 | 1.1 ± 0.1 |
| | Emergent adult | 0.5 ± 0.01$^{a}$ | 0.4 ± 0.05$^{ab}$ | 0.6 ± 0.02$^{b}$ | | Emergent adult | 1.3 ± 0.01 | 1.0 ± 0.2 | 1.1 ± 0.05 |
| | Young adult | 0.4 ± 0.02$^{a}$*** | 0.9 ± 0.1$^{b}$* | 0.6 ± 0.03$^{c}$ | | Young adult | 0.8 ± 0.03$^{a}$* | 1.9 ± 0.1$^{b}$*** | 1.6 ± 0.05$^{b}$ |
| | Old adult | 1.0 ± 0.1$^{a}$*** | 0.8 ± 0.05$^{ab}$ | 0.6 ± 0.02$^{b}$ | | Old adult | 1.5 ± 0.1*** | 1.7 ± 0.1 | 1.6 ± 0.07 |
| PC 16:1_18:1 | Early larva | 1.1 ± 0.1$^{a}$ | 0.7 ± 0.2$^{b}$ | 0.5 ± 0.01$^{a}$ | PE 16:1_18:1 | Early larva | 0.28 ± 0.04$^{a}$ | 0.17 ± 0.05$^{ab}$ | 0.12 ± 0.003$^{b}$ |
| | Late larva | 0.1 ± 0.01** | 0.2 ± 0.02*** | 0.3 ± 0.02 | | Late larva | 0.02 ± 0.002*** | 0.02 ± 0.002 | 0.05 ± 0.004 |
| | Pupa | 0.2 ± 0.03 | 0.1 ± 0.02 | 0.2 ± 0.01 | | Pupa | 0.01 ± 0.001 | 0.04 ± 0.003 | 0.03 ± 0.003 |
| | Emergent adult | 0.3 ± 0.01 | 0.3 ± 0.05 | 0.4 ± 0.01 | | Emergent adult | 0.05 ± 0.001 | 0.04 ± 0.01 | 0.05 ± 0.002 |
| | Young adult | 0.1 ± 0.01$^{a}$ | 1.6 ± 0.2$^{b}$*** | 1.5 ± 0.1$^{b}$*** | | Young adult | 0.03± 0.002$^{a}$ | 0.34 ± 0.05$^{b}$*** | 0.27 ± 0.01$^{b}$*** |
| | Old adult | 0.3 ± 0.02$^{a}$ | 1.0 ± 0.1$^{b}$ | 1.5 ± 0.1$^{c}$ | | Old adult | 0.07 ± 0.001$^{a}$ | 0.16 ± 0.01$^{a}$*** | 0.28 ± 0.03$^{b}$ |
| PC 18:1_18:1 | Early larva | 6.6 ± 0.7 | 6.0 ± 2.0 | 5.6 ± 0.2 | PE 18:1_18:1 | Early larva | 3.1 ± 0.3 | 2.7 ± 0.8 | 2.7 ± 0.1 |
| | Late larva | 4.4 ± 0.2*** | 3.2 ± 0.2* | 4.6 ± 0.2 | | Late larva | 1.7 ± 0.1$^{a}$*** | 0.8 ± 0.1$^{b}$*** | 2.0 ± 0.1$^{a}$ |
| | Pupa | 3.1 ± 0.2 | 3.0 ± 0.4 | 4.2 ± 0.3 | | Pupa | 1.0 ± 0.1 | 0.9 ± 0.1 | 1.2 ± 0.1 |
| | Emergent adult | 3.6 ± 0.1$^{a}$ | 5.2 ± 0.5$^{b}$* | 4.4 ± 0.1$^{b}$ | | Emergent adult | 2.0 ± 0.03** | 1.7 ± 0.4 | 1.7 ± 0.1 |
| | Young adult | 2.3 ± 0.1$^{a}$ | 6.8 ± 0.7$^{b}$ | 6.3 ± 0.2$^{b}$ | | Young adult | 2.0 ± 0.1$^{a}$ | 3.9 ± 0.3$^{b}$*** | 4.4 ± 0.3$^{b}$ |
| | Old adult | 5.2 ± 0.3*** | 7.1 ± 0.3 | 6.8 ± 0.2 | | Old adult | 3.4 ± 0.1$^{a}$*** | 3.2 ± 0.2$^{a}$ | 4.6 ± 0.2$^{b}$ |

Data are expressed in nmol· mg and as mean ± s.e.m. Letter indicates significant difference among the castes for the a given life-history stage with $p < 0.05$ (e.g. levels of PC 16:0_18:1 differ significantly among all caste at late larva stage. Asterisk indicates significant change from previous life-history stages within the same caste (i.e. level of PC 16:0_18:1 increases from late larva to pupa stage, in workers) with * $p < 0.05$ ,** $p < 0.01$, ***$p < 0.001$. Numbers of replicates per group are provided in Table 2.1.

Table 2. 5 – Main molecular phospholipids characterised in phosphatidylinositol (PIn) and phosphatidylserine (PS) of bee extract. Phospholipids in this table contain only saturated (SFA) and monounsaturated fatty acid (MUFA).

| | | Workers | Queens | Drones | | | Workers | Queens | Drones |
|---|---|---|---|---|---|---|---|---|---|
| PIn 16:0_18:1 | Early larva | 0.14 ± 0.03[a] | 0.14 ± 0.05[a] | ND | PS 16:0_18:1 | Early larva | 0.12 ± 0.01[a] | 0.09 ± 0.03[ab] | 0.07 ± 0.01[b] |
| | Late larva | 0.001 ± 0.0001*** | ND | ND | | Late larva | 0.05 ± 0.002*** | ND | 0.07 ± 0.01 |
| | Pupa | 0.0004 ± 0.0001 | ND | ND | | Pupa | 0.04 ± 0.002 | ND | 0.06 ± 0.01 |
| | Emergent adult | 0.02 ± 0.002 | ND | ND | | Emergent adult | 0.03 ± 0.001[a] | ND | 0.06 ± 0.001[b] |
| | Young adult | 0.005 ± 0.0004 | ND | ND | | Young adult | 0.01 ± 0.002[a] | ND | 0.05 ± 0.003[b] |
| | Old adult | 0.03 ± 0.01 | ND | ND | | Old adult | 0.03 ± 0.003 | ND | 0.04 ± 0.002 |
| PIn 18:0_18:1 | Early larva | 0.15 ± 0.03 | 0.13 ± 0.03 | 0.20 ± 0.02 | PS 18:0_18:1 | Early larva | ND | 0.010 ± 0.003 | ND |
| | Late larva | 0.002 ± 0.0003*** | 0.20 ± 0.03 | 0.14 ± 0.03 | | Late larva | ND | 0.003 ± 0.0002 | ND |
| | Pupa | 0.0003 ± 0.0001[a] | 0.32 ± 0.03[b] | 0.25 ± 0.09[b] | | Pupa | ND | 0.002 ± 0.0005[a] | 0.03 ± 0.01[b]*** |
| | Emergent adult | 0.05 ± 0.006[a] | 0.48 ± 0.1[b] | 0.25 ± 0.02[c] | | Emergent adult | 0.004 ± 0.0003[a] | 0.004 ± 0.001[a] | 0.04 ± 0.001[b] |
| | Young adult | 0.02 ± 0.0005[a] | 0.60 ± 0.1[b] | 0.04 ± 0.004[a]*** | | Young adult | 0.002 ± 0.0003[a] | 0.02 ± 0.003[b] | 0.01 ± 0.002[ab] |
| | Old adult | 0.04 ± 0.005[a] | 0.35 ± 0.04[b]*** | 0.05 ± 0.008[a] | | Old adult | 0.003 ± 0.0005 | 0.01 ± 0.001 | 0.01 ± 0.001 |
| PIn 16:1_18:1 | Early larva | ND | ND | ND | PS 16:1_18:1 | Early larva | 0.4 ± 0.04 | 0.3 ± 0.08 | 0.3 ± 0.1 |
| | Late larva | ND | ND | ND | | Late larva | 0.3 ± 0.02 | 0.3 ± 0.02 | 0.3 ± 0.04 |
| | Pupa | ND | ND | ND | | Pupa | 0.2 ± 0.01 | 0.2 ± 0.03 | 0.1 ± 0.1 |
| | Emergent adult | ND | ND | ND | | Emergent adult | 0.3 ± 0.01 | 0.4 ± 0.18 | 0.02 ± 0.001 |
| | Young adult | ND | ND | ND | | Young adult | 0.2 ± 0.01[a] | 0.5 ± 0.04[b] | 0.4 ± 0.01[ab] |
| | Old adult | ND | ND | ND | | Old adult | 0.3 ± 0.02 | 0.4 ± 0.03 | 0.3 ± 0.01 |
| PIn 18:1_18:1 | Early larva | 0.16 ± 0.02 | 0.1 ± 0.03 | 0.16 ± 0.01 | PS 18:1_18:1 | Early larva | 0.3 ± 0.03 | 0.3 ± 0.1 | 0.2 ± 0.04 |
| | Late larva | 0.001 ± 0.0002[a]*** | 0.2 ± 0.02[b] | 0.07 ± 0.02[c]*** | | Late larva | 0.2 ± 0.01[a]*** | 0.1 ± 0.01[a]*** | 0.2 ± 0.03[b] |
| | Pupa | 0.0003 ± 0.0001[a] | 0.3 ± 0.01[b]*** | 0.05 ± 0.01[a] | | Pupa | 0.2 ± 0.01 | 0.1 ± 0.02 | 0.1 ± 0.06 |
| | Emergent adult | 0.04 ± 0.004[a]*** | 0.4± 0.04[b] | 0.4 ± 0.01[b]*** | | Emergent adult | 0.2 ± 0.004* | 0.3 ± 0.15 | 0.02 ± 0.001 |
| | Young adult | 0.02 ± 0.001 | 0.006 ± 0.001*** | 0.04 ± 0.003*** | | Young adult | 0.1 ± 0.002[a] | 0.3 ± 0.03[b] | 0.3 ± 0.01[b]*** |
| | Old adult | 0.03 ± 0.003 | 0.004 ± 0.001 | 0.06 ± 0.01 | | Old adult | 0.2 ± 0.01[a]*** | 0.2 ± 0.02[a]*** | 0.2 ± 0.01[b] |

Data are expressed in nmol· mg and as mean ± s.e.m. Letter indicates significant difference among the castes for the a given life-history stage with $p < 0.05$ (e.g. levels of PIn16:0_18:1 are similar between workers and queens at early larva stage). Asterisk indicates significant change from previous life-history stages within the same caste (i.e. level of PIn 16:0_18:1 increases from early larva to late larva stage, in workers) with * $p < 0.05$ ,** $p < 0.01$, ***$p < 0.001$. Numbers of replicates per group are provided in Table 2.1.

#### *2.3.11.2 PUFA contains molecular phospholipids*

Tables 2.6 and 2.7 show the four most abundant phospholipid combinations that contain at least one PUFA (i.e. 16:0_18:3; 18:0_18:3, 18:1_18:2; 18:1_18:3) in PC and PE (Table 2.6), PIn and PS (Table 2.7). The level of phospholipid molecules that contained PUFA was low in all castes during early development. After emergence, the castes started to differ in their level of PUFA in the phospholipid molecules. In most cases, the castes reached their maximum level of PUFA-containing phospholipids as older adults. In PC, PE, and PS, old adult workers had the highest abundance of phospholipid molecules containing PUFA. However, adult queens showed a different pattern possessing some PIn molecules with higher PUFA abundance, notably PIn18:0_18:3.

The level of phospholipids containing PUFA in PC and PE was at least 3-fold higher in old adult workers than in old adult queens and drones (Table 2.6). In adult workers and queens, PC 18:1_18:3 was the most abundant PUFA-containing phospholipid whereas in drones PC 18:0_18:3 and PC 18:1_18:3 were the most common but at much lower levels. The most abundant PE phospholipid containing-PUFA in all castes were PE 18:0_18:3, PE 18:1_18:2 and PE 18:1_18:3. Old adult queens had a 2-fold higher level of PIn 18:0_18:3 and PIn 18:1_18:3 compared to both workers and drones (Table 2.7, $p < 0.05$). For PS, old workers had three times the level of PS 18:0_18:3 compared to old queens and drones. Drones as pupa had a higher level of PS 18:1_18:2 compared to pupa of queens and workers. Interestingly, the level of PUFA-containing phospholipids was more similar between queens and drones as adults, for all phospholipid classes except PIn.

Table 2. 6 – Main molecular phospholipids characterised in phosphatidylcholine (PC) and phosphatidylethanolamine (PE) of bee extract. Phospholipids in this table contain at least one polyunsaturated fatty acid (PUFA).

| | | Workers | Queens | Drones | | | Workers | Queens | Drones |
|---|---|---|---|---|---|---|---|---|---|
| PC 16:0_18:3 | Early larva | 0.04 ± 0.005 | ND | 0.03 ± 0.01 | PE 16:0_18:3 | Early larva | ND | ND | ND |
| | Late larva | 0.06 ± 0.005 | 0.02 ± 0.002 | 0.03 ± 0.01 | | Late larva | ND | ND | ND |
| | Pupa | 0.05 ± 0.004 | 0.01 ± 0.001 | 0.06 ± 0.01 | | Pupa | ND | ND | 0.01 ± 0.001$^{b}$ |
| | Emergent adult | 0.06 ± 0.003 | 0.02 ± 0.004 | 0.04 ± 0.004 | | Emergent adult | ND | 0.002 ± 0.001$^{a}$ | 0.004 ± 0.0004$^{ab}$*** |
| | Young adult | 0.2 ± 0.02*** | 0.1 ± 0.01* | 0.09 ± 0.02 | | Young adult | ND | 0.01 ± 0.001$^{a}$*** | 0.01 ± 0.001$^{b}$ |
| | Old adult | 0.4 ± 0.033$^{a}$*** | 0.1 ± 0.004$^{b}$ | ND | | Old adult | ND | 0.02 ± 0.002$^{a}$*** | 0.01 ± 0.001$^{b}$ |
| PC 18:0_18:3 | Early larva | ND | ND | ND | PE 18:0_18:3 | Early larva | ND | 0.02 ± 0.001 | 0.03 ± 0.002 |
| | Late larva | ND | 0.01 ± 0.001 | ND | | Late larva | ND | 0.02 ± 0.002 | 0.02 ± 0.002 |
| | Pupa | 0.02 ± 0.001 | 0.01 ± 0.001 | 0.02 ± 0.004 | | Pupa | ND | 0.02 ± 0.002 | 0.03 ± 0.005 |
| | Emergent adult | 0.04 ± 0.001 | 0.01 ± 0.003 | 0.02 ± 0.003 | | Emergent adult | 0.1 ± 0.003 | 0.02 ± 0.005 | 0.05 ± 0.005 |
| | Young adult | 0.08 ± 0.005$^{a}$ | 0.05 ± 0.004$^{a}$ | 0.4 ± 0.04$^{a}$*** | | Young adult | 0.2 ± 0.01* | 0.10 ± 0.01*** | 0.09 ± 0.01 |
| | Old adult | 0.35 ± 0.04$^{a}$*** | 0.06 ± 0.005$^{b}$ | 0.3 ± 0.02$^{a}$ | | Old adult | 0.5 ± 0.04$^{a}$*** | 0.12 ± 0.01$^{b}$ | 0.08 ± 0.005$^{b}$ |
| PC 18:1_18:2 | Early larva | 0.05 ± 0.01 | 0.06 ± 0.02 | 0.05 ± 0.002 | PE 18:1_18:2 | Early larva | 0.20 ± 0.02$^{a}$ | 0.03 ± 0.001$^{b}$ | 0.02 ± 0.001$^{b}$ |
| | Late larva | 0.14 ± 0.01 | 0.02 ± 0.003 | 0.05 ± 0.01 | | Late larva | 0.07 ± 0.01$^{a}$ | 0.003 ± 0.001$^{b}$ | 0.02 ± 0.001$^{b}$ |
| | Pupa | 0.10 ± 0.004 | 0.03 ± 0.004 | 0.16 ± 0.03 | | Pupa | 0.07 ± 0.003$^{a}$ | 0.01 ± 0.001$^{b}$ | 0.03 ± 0.004$^{b}$ |
| | Emergent adult | 0.12 ± 0.01$^{a}$ | 0.05 ± 0.01$^{a}$ | 0.40 ± 0.05$^{b}$*** | | Emergent adult | 0.04 ± 0.004$^{a}$ | 0.01 ± 0.003$^{a}$ | 0.07 ± 0.01$^{b}$ |
| | Young adult | 0.41 ± 0.02$^{a}$*** | 0.20 ± 0.01$^{b}$* | 0.03 ± 0.003$^{c}$*** | | Young adult | 0.16 ± 0.01$^{a}$*** | 0.07 ± 0.01$^{b}$*** | 0.10 ± 0.01$^{b}$ |
| | Old adult | 0.94 ± 0.06$^{a}$*** | 0.22 ± 0.02$^{b}$ | 0.04 ± 0.003$^{c}$ | | Old adult | 0.25 ± 0.01$^{a}$*** | 0.08 ± 0.01b | 0.10 ± 0.01$^{b}$ |
| PC 18:1_18:3 | Early larva | 0.11 ± 0.01 | 0.07 ± 0.002 | 0.16 ± 0.01 | PC 18:1_18:3 | Early larva | ND | 0.04 ± 0.004 | 0.06 ± 0.002 |
| | Late larva | 0.21 ± 0.02 | 0.07 ± 0.01 | 0.13 ± 0.01 | | Late larva | ND | 0.02 ± 0.002$^{a}$ | 0.05 ± 0.002$^{b}$ |
| | Pupa | 0.14 ± 0.01 | 0.04 ± 0.01 | 0.23 ± 0.1 | | Pupa | ND | 0.01 ± 0.002 | 0.04 ± 0.01 |
| | Emergent adult | 0.19 ± 0.01 | 0.07 ± 0.01 | 0.14 ± 0.01 | | Emergent adult | 0.07 ± 0.003$^{a}$ | 0.01 ± 0.004$^{b}$ | 0.04 ± 0.003$^{b}$ |
| | Young adult | 0.74 ± 0.04$^{a}$*** | 0.42 ± 0.04$^{b}$*** | 0.33 ± 0.04$^{b}$ | | Young adult | 0.26 ± 0.01$^{a}$*** | 0.14 ± 0.02$^{b}$*** | 0.12 ± 0.01$^{b}$ |
| | Old adult | 1.58 ± 0.12$^{a}$*** | 0.55 ± 0.06$^{b}$ | 0.25 ± 0.02$^{c}$ | | Old adult | 0.45 ± 0.03$^{a}$*** | 0.19 ± 0.03$^{b}$ | 0.11 ± 0.01$^{c}$ |

Data are expressed in nmol· mg and as mean ± s.e.m.. Letter indicates significant difference among the castes for the a given life-history stage with $p < 0.05$ (e.g. levels of PC 16:0_18:3 differ between workers and queens at old adult stage). Asterisk indicates significant change from previous life-history stages within the same caste (i.e. level of PC 16:0_18:3 increases from emergent adult to young adult stage, in workers) with * $p < 0.05$ ,** $p < 0.01$, ***$p < 0.001$. Numbers of replicates per group are provided in Table 2.1.

Table 2. 7 – Main molecular phospholipids characterised in phosphatidylinositol (PIn) and phosphatidylserine (PS) of bee extract. Phospholipids in this table contain at least one polyunsaturated fatty acid (PUFA).

| | | Workers | Queens | Drones | | | Workers | Queens | Drones |
|---|---|---|---|---|---|---|---|---|---|
| PIn 16:0_18:3 | Early larva | 0.04 ± 0.1 | ND | 0.03 ± 0.002 | PS 16:0_18:3 | Early larva | ND | ND | ND |
| | Late larva | 0.0005 ± 0.0001 | 0.06 ± 0.01 | 0.02 ± 0.004 | | Late larva | ND | ND | ND |
| | Pupa | 0.0003 ± 0.0001[a] | 0.05 ± 0.004[a] | 0.12 ± 0.03[b]*** | | Pupa | ND | ND | ND |
| | Emergent adult | 0.02 ± 0.002[a] | 0.02 ± 0.003[b] | 0.10 ± 0.01[b] | | Emergent adult | ND | ND | ND |
| | Young adult | 0.02 ± 0.002[a] | 0.2 ± 0.03[b]*** | 0.02 ± 0.003[a]*** | | Young adult | ND | ND | ND |
| | Old adult | 0.03 ± 0.005[a] | 0.11 ± 0.02[b]* | 0.02 ± 0.002[a] | | Old adult | ND | ND | ND |
| PIn 18:0_18:3 | Early larva | 0.06 ± 0.01 | ND | 0.1 ± 0.01 | PS 18:0_18:3 | Early larva | ND | ND | ND |
| | Late larva | 0.001 ± 0.0002 | 0.15 ± 0.02 | 0.06 ± 0.01 | | Late larva | 0.01 ± 0.002 | 0.004 ± 0.000 | ND |
| | Pupa | 0.001 ± 0.0002 | 0.11 ± 0.01 | 0.40 ± 0.1*** | | Pupa | 0.02 ± 0.002 | 0.007 ± 0.001 | 0.01 ± 0.004 |
| | Emergent adult | 0.20 ± 0.02[a]*** | 0.10 ± 0.01[a] | 0.60 ± 0.04[b] | | Emergent adult | 0.03 ± 0.001 | 0.005 ± 0.002 | 0.01 ± 0.000 |
| | Young adult | 0.09 ± 0.01[a] | 0.96 ± 0.2[b]*** | 0.11 ± 0.01[a]*** | | Young adult | 0.04 ± 0.003 | 0.03 ± 0.003 | 0.04 ± 0.003 |
| | Old adult | 0.30 ± 0.04[a]*** | 0.65 ± 0.1[b]*** | 0.10 ± 0.01[c] | | Old adult | 0.11 ± 0.01[a]*** | 0.04 ± 0.003[b] | 0.04 ± 0.002[b] |
| PIn 18:1_18:2 | Early larva | ND | ND | ND | PS 18:1_18:2 | Early larva | ND | ND | ND |
| | Late larva | ND | 0.01 ± 0.001 | ND | | Late larva | ND | ND | ND |
| | Pupa | ND | 0.03 ± 0.003 | 0.05 ± 0.02 | | Pupa | ND | 0.001 ± 0.000 | 0.15 ± 0.04*** |
| | Emergent adult | ND | 0.003 ± 0.003 | 0.09 ± 0.01 | | Emergent adult | 0.01 ± 0.001[a] | 0.004 ± 0.001a | 0.17 ± 0.005[b] |
| | Young adult | ND | 0.05 ± 0.01 | 0.03 ± 0.003 | | Young adult | 0.01 ± 0.002 | 0.01 ± 0.001 | 0.01 ± 0.001*** |
| | Old adult | ND | 0.04 ± 0.01 | 0.03 ± 0.003 | | Old adult | 0.02 ± 0.001 | 0.01 ± 0.000 | 0.01 ± 0.001 |
| PIn 18:1_18:3 | Early larva | ND | ND | 0.03 ± 0.003 | PS 18:1_18:3 | Early larva | ND | ND | ND |
| | Late larva | 0.004 ± 0.001 | 0.05 ± 0.01 | 0.02 ± 0.003 | | Late larva | 0.004 ± 0.001 | 0.003 ± 0.001 | ND |
| | Pupa | 0.01 ± 0.001[a] | 0.05 ± 0.005[a] | 0.13 ± 0.03[b]*** | | Pupa | 0.01 ± 0.001 | 0.004 ± 0.001 | 0.01 ± 0.003 |
| | Emergent adult | 0.02 ± 0.0004[a] | 0.04 ± 0.003[a] | 0.16 ± 0.01[b] | | Emergent adult | 0.02 ± 0.001 | 0.01 ± 0.001 | 0.01 ± 0.000 |
| | Young adult | 0.02 ± 0.001[a] | 0.20 ± 0.03[b]*** | 0.03 ± 0.003[a]*** | | Young adult | 0.02 ± 0.001 | 0.01 ± 0.001 | 0.02 ± 0.001 |
| | Old adult | 0.04 ± 0.002[a] | 0.16 ± 0.03[b] | 0.04 ± 0.005[a] | | Old adult | 0.04 ± 0.002[a]*** | 0.02 ± 0.003[b] | 0.02 ± 0.001[b] |

Data are expressed in nmol· mg and as mean ± s.e.m. Letter indicates significant difference among the castes for the a given life-history stage with $p < 0.05$ (e.g. levels of PIn 16:0_18:3 in workers and queens are different to drones at pupa stage). Asterisk indicates significant change from previous life-history stages within the same caste (i.e. level of PIn 16:0_18:3 increases from emergent adult to young adult stage, in queens) with * $p < 0.05$ ,** $p < 0.01$, ***$p < 0.001$. Numbers of replicates per group are provided in Table 2.1.

### 2.3.12 Plasmalogens

The level of plasmalogens for the various bee castes is shown in Table 2.8. Table 2.8 shows plasmalogens as non-PUFA or PUFA-containing and as total plasmalogens. For workers and queens, the total level of plasmalogens was low and similar during early development (i.e. EL, LL and PU). After emergence, workers increased total plasmalogen levels by ~2.8 fold and queens, by ~1.6 fold. Drones shared similar low levels of total plasmalogens with female castes at the early larvae stage but soon increased plasmalogen levels being higher than female castes in later life stages. Drones increased plasmalogen levels from late larva to pupation by ~7-fold (primarily made up of non-PUFA-containing plasmalogens). As adults, drones increased their level of plasmalogens (primarily from non-PUFA-containing plasmalogens) to possess the highest plasmalogen level of any caste at any life stage being 5-8 fold greater than workers and queens at the same life stage. As adults (with a few exceptions) the two female castes showed similar plasmalogen levels throughout their development. The major difference between workers and queens was that the increases in total plasmalogen levels, post-emergence, were primarily made up from increases in non-PUFA-containing plasmalogens for workers and by increases in PUFA-containing plasmalogens for queens.

Table 2. 8. Plasmalogens of whole bee extracts at different life-history stages of the three castes of honey bee (*Apis mellifera*).

| | Life Stages | Workers | Queens | Drones |
|---|---|---|---|---|
| Total | Early larva | $0.030 \pm 0.003^{a}$ | $0.030 \pm 0.006^{a}$ | $0.039 \pm 0.004^{a}$ |
| | Late larva | $0.013 \pm 0.002^{a}$*** | $0.010 \pm 0.001^{a}$ | $0.032 \pm 0.002^{b}$ |
| | Pupa | $0.051 \pm 0.007^{a}$** | $0.018 \pm 0.002^{b}$ | $0.223 \pm 0.133^{c}$** |
| | Emergent | $0.067 \pm 0.003^{a}$ | $0.096 \pm 0.014^{ab}$ | $0.130 \pm 0.004^{b}$ |
| | Young | $0.102 \pm 0.003^{a}$** | $0.170 \pm 0.012^{a}$** | $0.834 \pm 0.123^{b}$ |
| | Old | $0.187 \pm 0.008^{a}$** | $0.158 \pm 0.008^{a}$ | $1.020 \pm 0.092^{b}$ |
| Non PUFA | Early larva | $0.030 \pm 0.003^{a}$ | $0.027 \pm 0.003^{a}$ | $0.039 \pm 0.004^{b}$ |
| | Late larva | $0.013 \pm 0.002^{a}$*** | $0.010 \pm 0.001^{b}$ | $0.032 \pm 0.002^{c}$ |
| | Pupa | $0.028 \pm 0.004^{a}$** | $0.014 \pm 0.002^{ab}$ | $0.179 \pm 0.139^{b}$ |
| | Emergent | $0.060 \pm 0.002^{a}$*** | $0.032 \pm 0.004^{b}$ | $0.044 \pm 0.002^{c}$ |
| | Young | $0.085 \pm 0.003^{a}$*** | $0.053 \pm 0.004^{b}$** | $0.818 \pm 0.122^{c}$* |
| | Old | $0.150 \pm 0.006^{a}$*** | $0.055 \pm 0.005^{b}$ | $1.001 \pm 0.091^{c}$ |
| PUFA | Early larva | NA | NA | NA |
| | Late larva | NA | NA | NA |
| | Pupa | $0.020 \pm 0.003^{a}$*** | NA | $0.046 \pm 0.011^{a}$** |
| | Emergent | $0.010 \pm 0.001^{a}$ | $0.064 \pm 0.009^{b}$ | $0.082 \pm 0.004^{b}$ |
| | Young | $0.018 \pm 0.001^{a}$** | $0.118 \pm 0.009^{b}$** | $0.018 \pm 0.003^{a}$ |
| | Old | $0.035 \pm 0.003^{a}$* | $0.103 \pm 0.003^{b}$ | $0.017 \pm 0.002^{a}$ |

Plasmalogens were compiled for plasmalogen molecule phospholipid species into three categories: i) Plasmalogens non-PUFA that were associated with phospholipids with only saturated (SFA) and monounsaturated (MUFA) fatty acids, ii) plasmalogens PUFA that were associated with phospholipids with at least one PUFA and iii) total plasmalogens. Data are presented as mean ± s.e.m. and expressed as nmol of phospholipids· $mg^{-1}$ of bee. Letters (a, b, c) indicate a difference between the castes for the same plasmalogen category (e.g. Total plasmalogens was similar between early larva workers, early larva queens and early larva drones). Level of significance was set at $p < 0.05$ when comparing castes (e.g. early larva workers, early larva queens, early larva drones). Asterisks indicate a significant change from the previous life-history stage within the same caste (e.g. Total plasmalogens changed significantly from early larva to late larva in workers) with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. NA indicates where phospholipids were not present in the extract.

## 2.4 Discussion

The phospholipidome of the three castes (two female castes; workers, queens and one male caste: drones) of the honey bee, covering all life history stages (from small larva to old adults) was examined. The main findings of this study were that i) all castes have a very similar relative membrane fatty acid composition during their development (which is similar to adult queens). ii) After emergence as adults, workers increased their relative level of polyunsaturated fatty acids (PUFA) at the expense of monounsaturated fatty acids (MUFA) while queen and drone membranes remained highly monounsaturated. iii) The increase of

PUFA in adult worker membrane phospholipids occurred across most molecular phospholipid classes. Finally, iv) all castes maintained a similar level of the different phospholipid classes except for the level of phosphatidylinositol (PIn) that was higher in adult queens compared to the other castes.

The fatty acid composition of membrane phospholipids of honey bees was found to be relatively simple, with only six major fatty acids present i.e.16:0, 16:1, 18:0, 18:1, 18:2 and 18:3. The same fatty acids were previously reported in honey bees (Haddad et al., 2007; Robinson and Nation, 1970; Xu and Gao, 2013) and termites (Basalingappa et al., 1972). In agreement with these previous studies, the present study also observed a lack of longer chain fatty acids (longer than 18-carbon), including many common PUFA such as 20:4n-6 and 22:6n-3 found in the membranes of marine invertebrates (Munro and Blier, 2012; Kraffe et al., 2006) and vertebrates (Abbott et al., 2010, 2012; Andersson et al., 2002; Couture and Hulbert, 1995; Croset et al., 1989; Hulbert et al., 2002; Lemieux et al., 2008; McCue et al., 2009; Pan et al., 1995; Yamaoka et al., 1988). The membrane phospholipid fatty acid composition of honey bees was also found to be similar to those of other insects that have been measured including; fruit fly (Overgaard et al., 2005, 2008; Shen et al., 2010), butterfly (Wang et al., 2006), and chinch bug (Spike et al., 1991) as well as seven insect orders (Thompson, 1973). The lack of long chain fatty acids in honey bees, as well as in other insects, suggests an absence of these fatty acids in their diets as well as a lack of the desaturase and elongase enzymes necessary to produce longer chained PUFA *de novo* from their shorter chained precursors (i.e. 18:2 and 18:3).

All castes were found to have a very similar membrane fatty acid composition up to the pupation stage. As larvae, all bee castes were relatively highly monounsaturated (more than 70%, Figure 2.1-B) with very little polyunsaturation (less than 2%, Figure 2.1-C), however, from late-larvae to emergence, in both workers and drones, the level of PUFA increased, with compensatory reductions in MUFA. Queens did not display an increase in PUFA during pupation. The increase of PUFA in membrane phospholipids of both of the short-lived castes (i.e. workers and drones) suggests that workers and drones as larvae are partially feeding on pollen during this stage of their development (Tautz, 2008; Winston, 1987). In contrast, queens increase their level of MUFA during pupation with a

correspondent reduction in SFA in membrane phospholipids (Figure 2.1-A), suggesting future queens are not fed pollen during their larval stage.

Following emergence, adult castes started to differ significantly in the relative fatty acid composition of their membrane phospholipids. Workers increased their level of PUFA in their membrane phospholipids during the first week of their adult life (Figure 2.1-C), at the expenses of MUFA (Figure 2.1-B). This increase in PUFA was spread across most PUFA-containing molecules, particularly PC and PE in the membranes of workers (Table 2.6). This change reflected the progressive start of workers feeding on pollen following emergence as adults, although they are also fed liquid food mouth-to-mouth by older workers for the first week of adult life to complete their development (Sammataro and Avitabile, 1998; Tautz, 2008; Winston, M. L., 1987, Haydak, 1970). Pollen has a high abundance of PUFA (more than 50%, Table 2.2) and consequently, its consumption leads to a 3-fold increase in membrane PUFA during the first week of an adult workers life (transition from emergence to nurse stage). The membrane phospholipids of worker bees consequently remain relatively highly polyunsaturated throughout their adult life suggesting worker bees feed on pollen throughout their entire adult life. Only two PUFA are found in pollen: 18:2 and 18:3. These two PUFA are also the only two PUFA found in membrane phospholipids and triglycerides (data not shown) of worker bees. In contrast to the genetically identical female workers, the membrane phospholipids of queens remained relatively high in monounsaturates with very low levels of PUFA during their adult life. Queens are fed royal jelly, mouth-to-mouth by worker bees their whole life and are not normally observed eating pollen (Sammataro and Avitabile, 1998; Tautz, 2008; Winston, 1987). The low abundance of PUFA in the membrane phospholipids of queens (Haddad et al., 2007; Robinson and Nation, 1970; Xu and Gao, 2013) reflects the fatty acid composition of royal jelly i.e. very low level of PUFA, (Li et al., 2013; Xu and Gao, 2013) and thus strongly supports this hypothesis (to be discussed later).

### 2.4.1 Membrane composition, peroxidation, and ageing

Among lipids, one group has been implicated in ageing in term of producing damage to tissues that might influence longevity (Hulbert, 2005). These are the PUFA in membranes phospholipids that are susceptible to peroxidation (due to their content of *bisallylic* bonds as described in the introduction). In comparison, PUFA in monoglyceride, diacylglycerol or triglyceride appear to be slightly more resistant to peroxidation compare to PUFA in

phospholipids (Cosgrove et al., 1987) although the reason for this is currently unknown. Once peroxidation is initiated this process can damage surrounding lipids, proteins and DNA molecules (Hulbert, 2005). Empirical experiments have determined the susceptibility of different fatty acids in phospholipids to peroxidation and have demonstrated that 18:2 are 40x and 18:3 80x times more prone to undergo peroxidation when compared to a MUFA such as 18:1 (Cosgrove et al., 1987; Holman, 1954). Thus, when the relative fatty acid composition of a membrane is characterised, it is possible to calculate (as described in the section 2.2.5) its relative capacity to undergo lipid peroxidation independent of other influences (e.g. antioxidant status) and thereby its potential capacity to cause collateral damage. The membrane peroxidation index combines the relative abundance of different PUFA in membranes and their susceptibility to peroxidise (Hulbert, 2005). Therefore, the level of polyunsaturation and relative amount of PUFA within a membrane will determine how prone it is to peroxidation. The smaller the membrane peroxidation value, the more resistant the membrane is to peroxidative damage and vice versa.

The peroxidation index of membranes has been found to be inversely correlated to longevity in mammals and birds, across large ranges in body size from mouse to elephant (Hulbert et al. 2017; Hulbert et al., 2007). Membrane peroxidation index has also been correlated with differences in uniquely long-lived species such as the short-beaked echidna (*Tachyglossus aculeatus*) with a maximum lifespan of 54 years which based on body mass should live for a maximum lifespan of 14 years. The membrane peroxidation index also correlates with the 10-fold difference in longevity between the naked mole rat (*Heterocephalus glaber*; maximum lifespan of 32 years) and the similar-sized, mouse (*Mus musculus*, maximum lifespan of 3 years; Hulbert et al., 2006b). Naked mole rats have membranes that are very monounsaturated and subsequently peroxidation resistant compared to mice. Membrane peroxidation index is also inversely correlated with the maximum lifespan in invertebrates, such as bivalves mollusks that live-up to 500 years (Munro and Blier, 2012) and different strains of *Caenorhabditis elegans* that live for different periods of time (Shmookler Reis et al., 2011). The same relation has been reported in female honey bees (Haddad et al., 2007) and the results of the current study support these previous findings that queen honey bees possess peroxidation-resistant membranes based on their membrane phospholipid composition compared to workers, and this remains a potential explanation for the longevity of queens.

The present study found that all castes as larva shared similar membrane peroxidation index values, but by emergence as an adult, worker bees had a higher membrane peroxidation index value compared to queens (Figure 2.1-D). This result suggests the feeding of pollen during late larva to pupa stages. It has also been suggested that a compound found in pollen and honey i.e. *p*-couramic acid, provided to the larva of workers during their developing may inhibit some physiological processes such as ovary development (Mao et al., 2015) and therefore prevent a worker larva becoming a queen. Upon emergence, the membrane peroxidation index of workers increases a further 3-fold during the first week of adult life. Worker bees maintained this high membrane peroxidation index throughout their adult life with no significant changes observed between nurses and forager bees. Queens also increased their membrane peroxidation index after emergence but to a much less extent compared to workers. The membranes of an adult queen are much more monounsaturated compared to that of adult worker bees and thus far more resistant to peroxidation. As measured in the present study, queens maintained a low peroxidative potential of their membranes for up to three years, with no significant changes observed between 12 months and three-year-old queens.

These results suggest that the difference in the potential of membranes to peroxidise between adult female bees is more likely to be due to events that occur after they emerged as adults. Larvae from all castes, including drones, are fed similar food early on in their development. The larval food is produced by a combination of secretions from the mandibular and hypopharyngeal glands of worker bees (Winston, 1987). The food fed to future queens (royal jelly) is an approximate 1:1 mix of hypopharyngeal: mandibular glandular secretions while the food fed to worker larvae (worker jelly) is an approximate mix of 2:1 mix (Haydak, 1970; Winston, 1987). Royal jelly is a complex mixture consisting of amino acids, sugars, proteins and mineral salts with a low lipid content (3-10% of dry-mass; Ferioli et al., 2014; Xu and Gao, 2013). The fatty composition of royal jelly is mainly composed of free ether soluble fatty acids (not triglycerides or phospholipids). Most fatty acids are SFA or MUFA of short to medium chain length (under 20 carbons long) that are either hydroxylated in terminal or intermediate positions or as dicarboxylic fatty acids. There is essentially no PUFA in these secretions (reviewed in Li et al., 2013). One of the main components of royal jelly is the fatty acid 10 hydroxy-$\Delta_2$-decenoic Acid (10 HAD), which can comprise up to 6% of royal jelly (Barket al., 1959). This fatty acid has been proposed to

have an epigenetic role involved in the caste determination of honey bees (Spannhoff et al., 2011). The very similar membrane peroxidation index values of the female castes as larvae suggests that both types of jelly have the same or least very similar fatty acid compositions.

### 2.4.2 Contribution of phospholipids to the membrane peroxidation index of female honey bees

The major phospholipids containing PUFA in the female castes are found in phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylinositol (PIn) phospholipid classes (Figure 2.3). These phospholipids contribute relatively equally to membrane peroxidation index during larval stages and up to emergence. However, the contribution of the different classes of phospholipids changes significantly during adult life. Molecular phospholipid species from PC and PE make up to 85% of the membrane peroxidation index in adult workers while PC and PE contributed to less than 50% of membrane peroxidation index in adult queens (Figure 2.3). Interestingly, the castes have similar levels of PC (Figure 2.5-A) and PE (Figure 2.5-B) in adult life. Therefore, queens increase the abundance of phospholipids that contain SFA and MUFA (e.g. PC/PE 16:0_18:1; PC/PE 18:1_18:1) during their adult development. Such molecular phospholipids are more resistant to lipid peroxidation compare to phospholipids associated with PUFA and may act as antioxidant mechanisms in membranes by inhibiting the rate of peroxidation. For example, PC 16:0_18:1 that can delay the onset of peroxidation by increasing the lag phase of peroxidative reactions (delay the entry to the more damaging propagation phase) in experimental liposomes providing more time for antioxidants to reduce any peroxidation (Cortie and Else, 2015). Such mechanisms have yet to be explored in membrane isolated from animals. However, a similar mechanism for reducing peroxidation has been proposed for non-methylene interrupted PUFA found in molluscs. These extremely long-living bivalves have peroxidation resistant non-methylene interrupted fatty acids, where the double bonds are separated by more than one methylene, thus eliminating the peroxidation sensitive *bis-allylic* methylene normal found on PUFA molecules (Munro and Blier, 2012).

In adult queens, PIn phospholipid molecules containing PUFA contribute to 50% or more to membrane peroxidation index compared to less than 10% in adult workers (Figure 2.3) with adult queens having 4 to 6-fold higher level of PIn compared to adult workers (Figure 2.5-C). Adult queens have a particularly higher abundance of PIn 18:0_18:1, while

workers have very little of this molecule (Table 2.5). Such molecular phospholipids may help reduce lipid peroxidation using the same inhibition properties found for PC 16:0_18:1 (Cortie and Else, 2015). Low abundant phospholipid classes such as PIn might be involved in lipid and cell signaling as well as membrane trafficking (Di Paolo and De Camilli, 2006). PIn phospholipids may also be involved in other physiological processes such as pheromone production in queens. Data regarding the implication of different phospholipid classes and their effects on physiological processes and lipid peroxidation is scarce and insufficient to make any generalization on their implication to ageing (Schnitzer et al., 2007).

Plasmalogens differ from regular phospholipids in that the carbon chain of the sn-1 position is linked to the glycerol backbone by a vinyl-ether linkage, instead of the regular ester-linkage. This change in structure has been proposed to allow these molecules to act as a ROS scavenger that can potentially stop lipid peroxidation and confer antioxidant protection to membranes (Engelmann, 2004). Interestingly, naked mole rats with their 10-fold difference in maximum lifespan compared to normal rats have a higher abundance of plasmalogens as PE (PEP) molecular phospholipids (Mitchell et al., 2007). However, such comparisons on invertebrates are very limited. Molluscs show an inconsistent association (although only a few species have been examined) between the abundance of plasmalogens and longevity (Munro and Blier, 2012). In honey bees, the level of plasmalogens was very similar during development between the three castes. However, their abundance increases after emergence in all caste but to different extents. Both female castes had a similar level of plasmalogens as adults while adult drones had 10-fold higher level of plasmalogens compared to the female castes. The fatty acid composition of these plasmalogens was very different between the castes. In adult drones and adult workers most of the plasmalogens (> 80%) were associated to SFA and MUFA whereas, in adult queens, more than 60% of plasmalogens were associated with PUFA, 5-fold higher in queens compared to workers and drones (Table 2.8). A higher abundance of plasmalogens associated with PUFA, together with a lower level of PUFA in adult queens, suggests that queens may use plasmalogens as a means of protecting PUFA incorporated into their membranes. Support for such a mechanism is the high level of plasmalogens associated with PUFA in emergent drones. Interestingly, drones had a higher level of PUFA when emerging as adults that suggests drones may use plasmalogens for protecting PUFA incorporated into their membrane phospholipids. However, it is important to acknowledge that the method used in the current study could not distinguish plasmalogens

from isomeric alkyl-ether species by identification of the double-bond position (e.g. PEP 18:1_18:2 versus PE-O-18:1_18:2 will be recognised as the same molecules using the current method). The methods used may also underestimate the abundance of plasmalogens in PE by up to 30% (Abbott et al., 2013; Mitchell et al., 2007; Zemski Berry and Murphy, 2004). Further investigation would be required to confirm the exact composition of those phospholipids. However, the important information here is that whether they were plasmalogen or alkyl ether, alkyl ether (=O) phospholipids associated to MUFA would also contribute to reducing peroxidation as previously discussed.

### 2.4.3 Is the difference in membrane peroxidation index between female castes enough to explain the difference in lifespan in female honey bees?

In mammals and birds, a 20-25% decrease in membrane peroxidation index of skeletal muscle and liver mitochondria phospholipids is correlated with a 2-fold extension of lifespan (Hulbert et al., 2017). Assuming a similar linear relationship in honey bees, the 3-fold difference in membrane peroxidation index between adult workers and adult queens would be associated with an 8-16-fold difference in maximum lifespan between the castes. Giving that the maximum lifespan of worker bees is 140 days (an age data base), the expected maximum lifespan of a queen will be between 3-6 years. The maximum lifespan of the longest-living queen is currently 8 years (An age data base). Furthermore, when the membrane peroxidation index of the castes is plot on a log-log scale with maximum lifespan other animals, the exponent from the equation of best fit is very similar to animals previously reported in the literature (0.23 in honey bees versus 0.26-0.30 for mammals and 0.29 for invertebrates; Hulbert et al., 2017). Overall, the difference in the membrane peroxidation index between the castes appears sufficient, in theory, to partly explain the difference of maximum longevity between female castes.

### 2.4.4 Male versus females

An unexpected finding in this study was the low abundance of PUFA in membrane phospholipids of adult drones. In contrast to female workers, the relative level of PUFA decreased after emergence in male drones (Figure 2.1-C) to reach a level lower than queens. This reduction in membrane PUFA in adult drones is associated with a correspondent increase in MUFA (Figure 2.1-B) in adult drones. The low abundance of PUFA in membrane phospholipids of drones supports the proposal that drones are fed on food other than pollen

during their adult life. Literature suggests that drones feed on pollen like worker bees (Sammataro and Avitabile, 1998; Tautz, 2008; Winston, 1987), but this seems not supported by the present results showing a reduction of PUFA in membrane phospholipids of adult drones compared to emergent drones. An alternative hypothesis suggested by the current results is that drones are dependent on workers to be fed royal jelly, just like queens.

The membrane fatty acid composition of adult drones produces a very low membrane peroxidation index value compared to adult worker bees. As found in queens, drones have highly monounsaturated membranes that would make them highly resistant to lipid peroxidation. This finding seems to go against the prediction of the influence of the membrane peroxidation on lifespan, given that drones are known to have a short lifespan (Rueppell et al., 2005). Adult drones also have a reduced level of plasmalogens associated with PUFA (like workers; Table 2.8) compared to long-living queens. The level of plasmalogens in drones increased from larva to emergence to reach levels similar to those of emergent queens (Table 2.8). However, the level of plasmalogens associated with PUFA reduces rapidly after emergence as adults. In social insects, males accomplish no specific tasks within the colony, except providing their sperm for the survival of the species. In *Hymenoptera,* such as honey bees, males start their sexual life with a fixed amount of sperm sufficient for one insemination because their testes start to degrade before they emerge as adults (Moors et al., 2009). Consequently, there is no clear advantage for the colony to maintain drones that cannot replenish their sperm supplies. In honeybees, as for many species of ants, males typically live for a few days as copulation is suicidal and male die during or shortly after inseminating a queen. In most social insects the lifespan of males is not correlated to the lifespan of queens but rather appears to be adapted to mating opportunities. For example, *Cardiocondyla* ants colonies have multiple queens and long-living males that have a lifespan similar to those of queens (many months; Yamauchi et al., 2006). Another interesting example of long-living males is termite kings with a lifespan matching that of long-living queens (Judith Korb and Barbara Thorne, 2017). In honey bees, the relatively single queen may influence the behaviour of workers to remove drones from the hive, which will drastically shorten their lifespan. Therefore, based on the low membrane peroxidation index of adult male honey bees their lifespan could be much longer than that which naturally occurs if they continued to be maintained (e.g. feed) by workers in the hive.

### 2.4.5 Honey bee queens versus other long-lived organisms

An interesting perspective emerging from this research is a similarity between honey bee queens and the longest living rodent known: the naked mole rat. The naked mole rat is similar in size to the mouse but lives for up to 10-fold longer (Buffenstein and Jarvis, 2002; maximum lifespan is 31 years). Naked mole rats share very similar membrane features compared to honey bee queens, that is membranes resistant to peroxidation (i.e. low PUFA content, low membrane peroxidation index) and high level of plasmalogen molecules compared to short-living rodents (Hulbert et al., 2006b; Mitchell et al., 2007). Naked mole rats have also high abundance of molecular phospholipids resistant to peroxidation (i.e. PC/ PE 18:0_18:1; PC/ PE16:0_18:1; Mitchell et al., 2007) that could act as potential antioxidants (Cortie and Else, 2015). Naked mole rats carry high levels of oxidative damage, show reduced antioxidant defenses but higher protein stability with age together with increase proteasome activity (Buffenstein, 2005; Rodriguez et al., 2011b; Sohal and Orr, 2012). They also share characteristics with calorie-restricted mice models in addition to reduced incidence of cancer compared to rats (Buffenstein, 2005). Such alternative model has gained more interest over the last decade by gerontologists and reveals that longevity can be achieved through mechanisms going against theoretical and experimental predictions.

## 2.5 Conclusions

This work has produced a comprehensive analysis of the phospholipidome of all three castes of honey bees at all stages of their lives using bees from a free-living hive. All honey bee castes were found to possess very similar membrane phospholipid fatty acid compositions during development (larvae and pupae). The membrane phospholipid fatty acid composition differed significantly between sterile female workers and reproductive queens once they emerged as adults. These differences in membrane phospholipid composition appear to be based on nutritional differences between adult workers and queens. Workers feed on pollen once they emerge whereas queens are fed royal jelly by workers and appear not to eat pollen. The consumption of pollen by worker bees is associated with an increase in the relative level of polyunsaturated fatty acids (PUFA) in their membrane phospholipids. This increase in PUFA leads to membranes that are potentially more susceptible to peroxidation. In contrast, the membrane phospholipids of queens remain highly monounsaturated and resistant to oxidative damages. Adult queens also have a higher

abundance of plasmalogens associated with PUFA-containing phospholipids compared to short-living workers. Thus, the current results suggest that the extraordinary lifespan of queens is partially explained by queens avoiding pollen rather than the consumption of royal jelly. In other words, it is not what is in royal jelly but rather what the queen is avoiding (i.e. PUFA in pollen) that may help to explain the difference in lifespan between the female castes. The similar membrane peroxidation index between emergent workers and old queens (3 years) suggests that emergent worker bees are an excellent model to test the causation effect of membrane peroxidation index on longevity. Drones as adults remain an enigma as they possess membranes with a low peroxidation index yet live short-lives. This could be explained by the dependence of drones on workers for their maintenance and their forced removal from the hive when they are no longer required.

# Chapter 3. Membrane phospholipid regulation during adult life of worker bees.

# 3.1 Introduction

Two contrasting female phenotypes can be produced from the identical genome in honey bees (workers and queens) as a result of biological constraints not yet to be fully understood (Ashby et al., 2016). Among many differences between the castes (i.e. behaviours, pheromones, and anatomy) one of the most intriguing is the extension in lifespan present in queens compared to worker bees. Queens live for years while workers live for weeks. Over their relatively short lifespans, adult worker bees evolve through a series of different behavioral life-history stages (see details in section 1.6.1 and review in Winston, 1987). The two female castes also consume different foods during their adult life. After emerging workers feed on pollen (high in polyunsaturated fatty acids; PUFA) whilst queens are fed royal jelly (low in PUFA) throughout their adult life (Haydak, 1970). Worker bees consume pollen in the form of 'bee-bread' (the pollen packed into honeycomb by worker bees; Ellis and Jr, 2009; Haydak, 1970; Pernal and Currie, 2000; Tautz, 2008; Winston, 1987). The different diets of the adult female castes correspond with changes in the fatty acid composition of cellular membranes. Young adult worker bee membranes become more polyunsaturated at the expense of monounsaturated fatty acids (see sections 2.3.4 and 2.3.5). In contrast, the membranes of queens remain highly monounsaturated throughout their adult life as noted in previous studies (Haddad et al., 2007; Robinson, 1990).

The results of Chapter 2 show that nurse bees (aged approximately 7-14 days) have increased levels of membrane PUFA compared to that at emergence, and that forager bees (aged approximately 14+ days) have a similar proportion of PUFA in their membranes compared to nurse bees. These results suggest that changes in membrane fatty acid composition are happening between emergence and day 7-14 of adult worker life (corresponding to nurse bees). To further understand these changes in the fatty acid composition of membrane phospholipids, a detailed characterisation of membrane phospholipids of adult workers at different ages was undertaken.

# 3.2 Methods

### 3.2.1. Honey bees

A full frame of worker bee brood was collected from a hive maintained at the

University of Wollongong during the austral summer of 2015. The frame was placed overnight in an incubator at 34 ± 0.5 °C with a relative humidity of 60%. As they emerged from their cell in the honeycomb, worker bees were collected and marked with an acrylic marker (Arline®400XF). Marked bees were gently brushed off the frame, pooled inside a cardboard cylinder for reintroduction back to their original hive. A couple of puffs of smoke were used to facilitate the re-introduction of marked worker bees, reducing the potential for rejection (Münch et al., 2013). Marked worker bees were then subsequently collected at 0 day (emergence), 1, 2, 4, 7, 14 and 21 days of age. Once collected bees were immediately frozen and stored at -80°C until analysed.

### 3.2.2 Molecular phospholipids

Whole bee phospholipids were characterised using mass spectrometry as previously described (section 2.2.3). Phospholipids were analysed in 6 different headgroups phosphatidylcholine (PC), lysophosphatidylcholine (LPC), phosphatidylethanolamine (PE), lysosphosphatidylethanolamine (LPE), phosphatidylserine (PS) and phosphatidylinositol (PIn). Phospholipids were further characterised as non-PUFA and PUFA-containing phospholipids, as lysophospholipids and as the total for phospholipid molecules. Non-PUFA phospholipids were defined as the sum of all phospholipid molecules that contained only saturated (SFA) and monounsaturated fatty acids (MUFA) fatty acids, whereas PUFA-containing phospholipids were defined as those phospholipid molecules that contained at least one PUFA. Total lysophospholipids are the sum of all lysophospholipids characterised in bee extract. Total phospholipids were calculated as the sum of all phospholipid molecules characterised (i.e. Non-PUFA + PUFA + lysophospholipids). The list of all phospholipid molecules in the different categories found in bee extract is shown in Appendix 3. Phospholipids are expressed as nmol. $mg^{-1}$ of bee.

### 3.2.3 Membrane Fatty acids

Membrane fatty acid composition was calculated from total phospholipid quantified by Lipidview™ using a formulated Excel spreadsheet (Microsoft Corporation, WA, USA) that accounted for both the proportion of each phospholipid (i.e. its contribution to total phospholipids) together with its fatty acids composition as previously outlined (2.2.4). Fatty acids are expressed as % of total fatty acid.

### 3.2.4 Peroxidation index

The membrane peroxidation index of whole bee lipid extract was calculated from the total membrane phospholipid fatty acid composition data, as previously outlined (2.2.5).

### 3.2.5 Pollen fatty acids

The fatty acid composition of pollen was determined from samples of 'bee-bread' taken from comb cells on the same frame as used to sample worker bees. Pollen samples from different cells were taken, pooled and mixed. Aliquots of 'bee-bread' (500 mg, $n$ = 3) were weighted (±0.01 mg), placed in glass vials and then mixed with 10 volume of methanol (MeOH): tertiary butyl methyl ether (MTBE; 1:2) with 0.01 % BHT, 300 ul of 150mM Ammonium acetate and 100 µg of 23:0 fatty acid as an internal standard. Vials were capped under nitrogen and gently inverted over 24 hours. Samples were then spun at 2,000 g for 10 minutes and the upper lipid fraction collected then dried under nitrogen before transmethylation. The total lipid fraction was transmethylated for 60 min at 100°C after addition of 800 µl of acetyl chloride (Sigma Aldrich, NSW, Australia). After cooling, and adding 5mL of potassium carbonate (Sigma Aldrich, NSW, Australia), the organic phase-containing the fatty acid methyl esters were recovered. Fatty acid methyl esters (FAME) were identified and quantified using gas chromatography (GC) (Shimadzu GC-17A, NSW, Australia) using a VARIAN Fused Silica Column (50m X 0.25 mm ID, CP7419, NSW, Australia) with the following temperature program: 150°C initial temperature, 17.5°C/min to 170 °C, 0.5°C/min to 178 °C, 15°C min to 222°C and 2°C/min to 232°C. The split ratio was set at 25:1 for all analyses. Individual fatty acids were identified by comparison with an external standard (FAME mix C8-C24 Sigma Aldrich, NSW, Australia), quantified as nmol. $mg^{-1}$ of pollen using Shimadzu class-VP software™ (Shimadzu, 2002) and expressed as mol % of total fatty acids.

### 3.2.6 Statistical analysis

Membrane phospholipids of adult workers were compared against ageing and using emergent as the reference group (e.g. one-day-old bee versus emergent bee; 14-day-old bee versus emergent bee). An analysis of variance (ANOVA) using age as factor with Tukey post-hoc was used to compare membrane phospholipids of adult workers. Non-parametric Mann-Whitney $U$ tests was used if the data were not normally distributed. All analyses were

performed with R software (Version 3.2.2).

# 3.3 Results

### 3.3.1 Pollen fatty acids

The fatty acids composition of 'bee-bread' pollen is presented in Table 3.1 with the five most abundant fatty acids shown in Figure 3.1. The main five fatty acids of pollen accounted for more than 80% of total fatty acids and were the only major fatty acids found in bees (i.e. 16:0, 16:1, 18:0, 18:1, 18:2, 18:3). Overall, pollen was composed of 39% saturated (SFA), 14% monounsaturated (MUFA) and 47% polyunsaturated (PUFA) fatty acids. The three main fatty acids that dominated the composition of 'bee-bread' pollen were palmitic (16:0), linoleic (18:2) and linolenic (18:3) fatty acid. All three of these fatty acids were found in similar proportion (23-25%) with 18:2 and 18:3 the only two PUFA molecules present in pollen.

Table 3. 1. Relative percent fatty acid composition of bee-bread pollen

| **Fatty acids** | **Mol %** |
|---|---|
| 14:0 | 3.2 ± 0.1 |
| 14:1 | 2.0 ± 0.1 |
| 15:0 | 6.4 ± 0.2 |
| 15:1 | 0.5 ± 0.3 |
| 16:0 | 23.6 ± 0.3 |
| 16:1 | Trace |
| 17:0 | 3.2 ± 0.3 |
| 17:1 | 1.5 ± 0.1 |
| 18:0 | 1.8 ± 0.1 |
| 18:1 | 5.8 ± 0.1 |
| 18:2 | 25.2 ± 0.3 |
| 18:3 | 22.7± 0.1 |
| 20:0 | 0.3 ± 0.2 |
| 20:1 | 3.8 ± 0.1 |
| Total SFA | 38.5 ± 0.2 |
| Total MUFA | 13.6 ± 0.5 |
| Total PUFA | 47.9 ± 0.3 |
| Peroxidation index | 70.5 ± 0.3 |

Data are expressed as mol % of total fatty acids, values are mean ± s.e.m., $n$ = 3. See section 3.2.5 for more details.

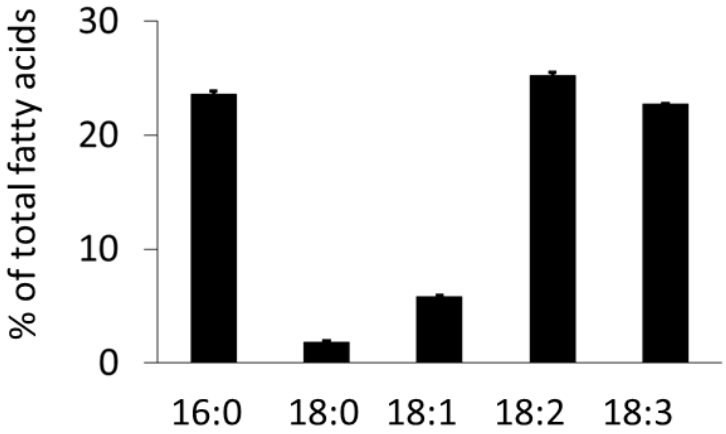


Figure 3. 1. Main fatty acids in total lipid extracts of bee bread pollen (see section 3.2.5 for details). *N* = 3, data are expressed as mean ± s.e.m. and as percent of total fatty acids.

### 3.3.2 Molecular phospholipids

The amount of membrane i.e. the concentration of phospholipid molecules per mg of bee increased rapidly (70%) during the first 24 hours of adult life following emergence increasing from 10 to 17 nmol. $mg^{-1}$ of bee (Figure 3.2). This increase in phospholipids was then followed by a small but insignificant decrease that saw total molecular phospholipid concentration settle at an intermediate level between emergent and one-day-old worker bees. Among the different types of phospholipids, non-PUFA-containing phospholipids increased rapidly in the first 24 hours post-emergence. This increase accounted for the overall increase in total phospholipids during this early period of adult development, as neither the PUFA-containing phospholipids or lysophospholipids increased in the first 24 hours post-emergence (Figure 3.2). Following this immediate increase, which peaked on day 1, non-PUFA-containing phospholipids then decreased in concentration over the next several days to settle at emergence levels by day 4, and they remained at this level for the rest of adult life. The PUFA-containing phospholipids also increased significantly ($p < 0.001$) in their concentration (5-fold) but this change was slower, not peaking until approximately day 4. However, this increase in PUFA-containing phospholipids was sustained for the remaining 17 days of measurement, resulting in the descending level of total phospholipids to recover and settle at an intermediate level between emergence and the peak on day 1. In contrast, the level

of total lysophospholipids remained low and relatively stable throughout adult life in workers.

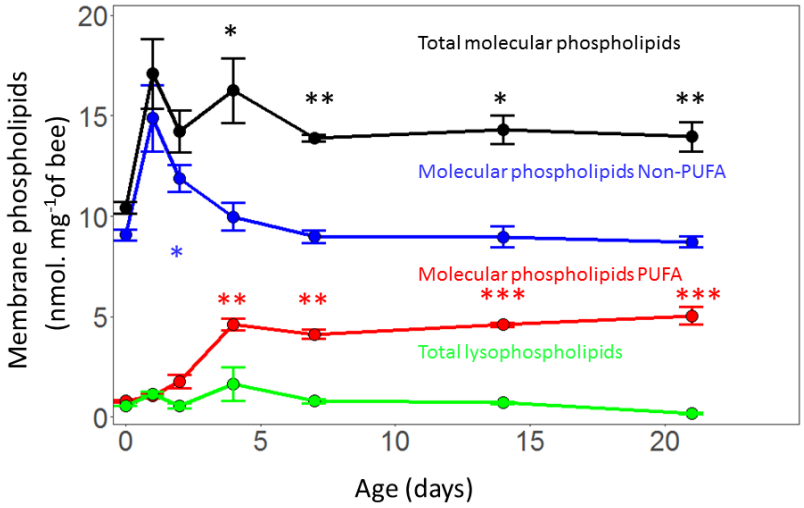


Figure 3. 2. Level of phospholipid molecules in the membranes of worker bees at different ages. Ages characterised were emergent (0), 1, 2, 4, 7, 14 and 21 days. Data are presented as mean ± s.e.m. and expressed as nmol of phospholipid. $mg^{-1}$of bee. Where error bars are not obvious, error bars are less than the size of the marker. $N = 3$ for each age. See appendix 3 for the list of different categories of phospholipid molecules. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ indicate the level of significant difference from emergent worker bees.

The main types of phospholipids found in bees (shown in Table 3.2) were non-PUFA-containing phospholipids of which PC 18:1_18:1 was the most abundant followed by PE 18:1_18:1, PC 16:0_18:1 and PE 18:0_18:1. Two phospholipids, PC 18:1_18:1 and PE 18:1_18:1 primarily accounted for 18:1 being the major fatty acid of worker bees (Figure 3.4). Increases in the non-PUFA-containing phospholipids accounted for an immediate increase in phospholipid concentration in worker bees in the first 24 hours following emergence, with the greatest contribution coming from PC 18:1_18:1 (at 1.8 nmol. $mg^{-1}$) and the greatest relative contribution from PC 16:0_18:1 that doubled in its relative amount (Table 3.2). Following the initial rise in non-PUFA-containing phospholipids, there was a subsequent decline in all four phospholipids. However, a slower but persistent increase in PUFA-containing phospholipids up to day 4, primarily driven by increases in the level of PC 18:1_18:2 (11-fold increase) and to a much lesser extent by PC 18:1_18:3, PC 18:2_18:2, PC 16:0_18:2 and PE 18:0_18:2. The increase in these PUFA-containing phospholipids slowed the decline in total phospholipids to produce steady-state levels by day 7 (Table 3.2).

Table 3. 2. Major phospholipid molecules in worker bee extracts.

| Type of phospholipids | Molecule | Emergent | 1 day | 2 days | 4 days | 7 days | 14 days | 21 days |
|---|---|---|---|---|---|---|---|---|
| Non PUFA | PC 18:1_18:1 | 3.00 ± 0.13 | 4.81 ± 0.43* | 3.87 ± 0.26 | 2.83 ± 0.25 | 2.64 ± 0.22 | 2.78 ± 0.24 | 2.30 ± 0.13 |
| | PE 18:1_18:1 | 1.51 ± 0.06 | 2.57 ± 0.18*** | 2.46 ± 0.14** | 1.90 ± 0.10 | 1.94 ± 0.09 | 2.06 ± 0.07 | 1.75 ± 0.65 |
| | PC 16:0_18:1 | 1.06 ± 0.05 | 2.14 ± 0.33** | 1.36 ± 0.03 | 1.01 ± 0.10 | 0.98 ± 0.79 | 0.70 ± 0.04 | 0.67 ± 0.02 |
| | PE 18:0_18:1 | 0.86 ± 0.05 | 1.16 ± 0.07 | 1.03 ± 0.06 | 0.86 ± 0.06 | 0.79 ± 0.08 | 0.82 ± 0.01 | 0.61 ± 0.24 |
| PUFA | PC 18:1_18:2 | 0.13 ± 0.02 | 0.23 ± 0.03 | 0.49 ± 0.11*** | 1.41 ± 0.06*** | 1.37 ± 0.07*** | 1.19 ± 0.05*** | 2.00 ± 0.16*** |
| | PC 18:1_18:3 | 0.07 ± 0.01 | 0.12 ± 0.01 | 0.20 ± 0.05 | 0.30 ± 0.01*** | 0.22 ± 0.02*** | 0.21 ± 0.01*** | 0.24 ± 0.03*** |
| | PC 18:2_18:2 | Trace | Trace | 0.03 ± 0.01* | 0.30 ± 0.01*** | 0.22 ± 0.02*** | 0.22 ± 0.01*** | 0.24 ± 0.03*** |
| | PC 16:0_18:2 | Trace | 0.04 ± 0.01 | 0.09 ± 0.01** | 0.30 ± 0.02*** | 0.22 ± 0.02*** | 0.23 ± 0.01*** | 0.21 ± 0.01*** |
| | PE 18:0_18:2 | 0.02 ± 0.01 | 0.03 ± 0.01 | 0.08 ± 0.01** | 0.26 ± 0.01*** | 0.22 ± 0.01*** | 0.34 ± 0.01*** | 0.15 ± 0.01*** |
| | PE 18:1_18:3 | 0.04 ± 0.01 | 0.06 ± 0.01 | 0.09 ± 0.01 | 0.23 ± 0.01*** | 0.25 ± 0.03*** | 0.27 ± 0.01*** | 0.57 ± 0.04*** |

*N* =3 for each age, data are presented as mean ± s.e.m. and expressed as nmol. $mg^{-1}$ of bee. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ indicate the level of significant difference from emergent worker bees.

### 3.3.3 Plasmalogens

The level of plasmalogens of worker bees constantly increased during adult life reaching a maximum level in three-week-old workers (Figure 3.3). In general, worker bees had a higher level of plasmalogens associated with SFA and MUFA compared to plasmalogens associated with PUFA at any age. Levels of plasmalogens were also fairly small compared to the total amount of phospholipid and accounted for only about 1-2 % of total phospholipid content. As previously mentioned in Chapter 2, the exact position of the double bond could not be determined on plasmalogen phospholipids using the current method and, therefore, the current plasmalogen molecules could also be alkyl-ether phospholipids linked to a MUFA (i.e. O=MUFA).

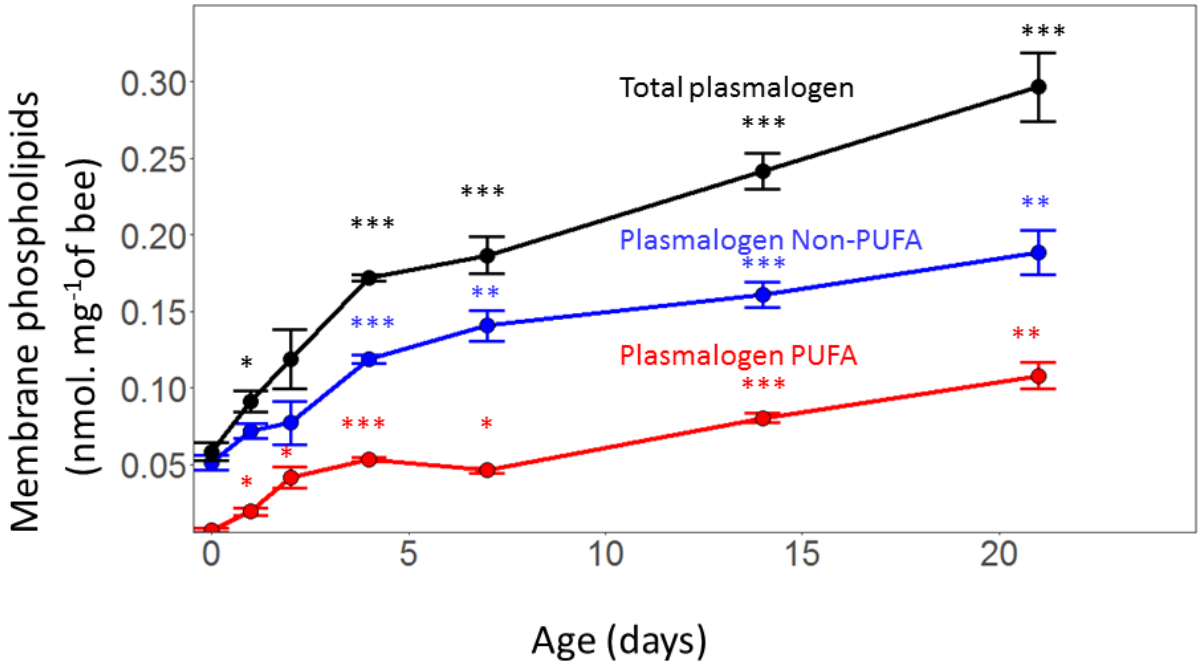


Figure 3. 3. Plasmalogen levels in membrane phospholipids of adult worker bees. Ages characterised were emergent (0), 1, 2, 4, 7, 14 and 21 days. Data are presented as mean ± s.e.m. and expressed as nmol of phospholipids. $mg^{-1}$of bee. Where error bars are not obvious, error bars are less than the size of the marker. $N = 3$ for each age. See appendices 3 for the list of different categories of phospholipid molecules. * $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$ indicate the level of significant difference from emergent worker bees.

### 3.3.4 Membrane phospholipid fatty acids

Six (6) main fatty acids were quantified following the analysis of phospholipids isolated from adult worker bees. These fatty acids were the same fatty acids reported in Chapter 2 i.e. 16:0, 16:1, 18:0, 18:1, 18:2, 18:3. The relative level of each of these fatty acids over the first 21 days of adult life is presented in Figure 3.4. The two main saturated fatty acids (SFA) 16:0 and 18:0, each contributing to ~10% of total fatty acid showed very similar trends during ageing with both remaining relatively low and constant throughout adult life (although 16:0 did decrease significantly at 14 and 21 days). Amongst MUFA, 18:1 was by far the most abundant fatty acid found in the membrane phospholipids of workers. The level of 18:1 decreased progressively from 75% of total fatty acids in emerging worker to reach its lowest level at 58% of total fatty acids by day 4 where it remained for the rest of in adult life. A second but far less prevalent MUFA was 16:1 (4% of total fatty acid) that showed some small significant changes.

Only two PUFA, 18:2 and 18:3, were found to be present in the membrane phospholipids of worker honey bees. Both showed very similar trends during ageing with very low abundance upon emergence (0.5 and 2.5% of total fatty acids for 18:2 and 18:3, respectively) followed by significant increases occurring over the first four days post-emergence for both 18:2 and 18:3 (Figure 3.4). For 18:2 a maximum value of 12.5% of total fatty acids was reached after four days whereas in the case of 18:3 a maximum value was reached at two weeks (at 7.5% of total fatty acids). Levels of 18:2 and 18:3 thereafter remained steady at these higher levels for the duration of the measurement period, with the PUFA level of membranes increasing 7-fold during adult life (from 3% at emergence to 20% of total fatty acids in old adults). The increase in total PUFA came at the expense of the MUFA 18:1 that decreased by 17%.

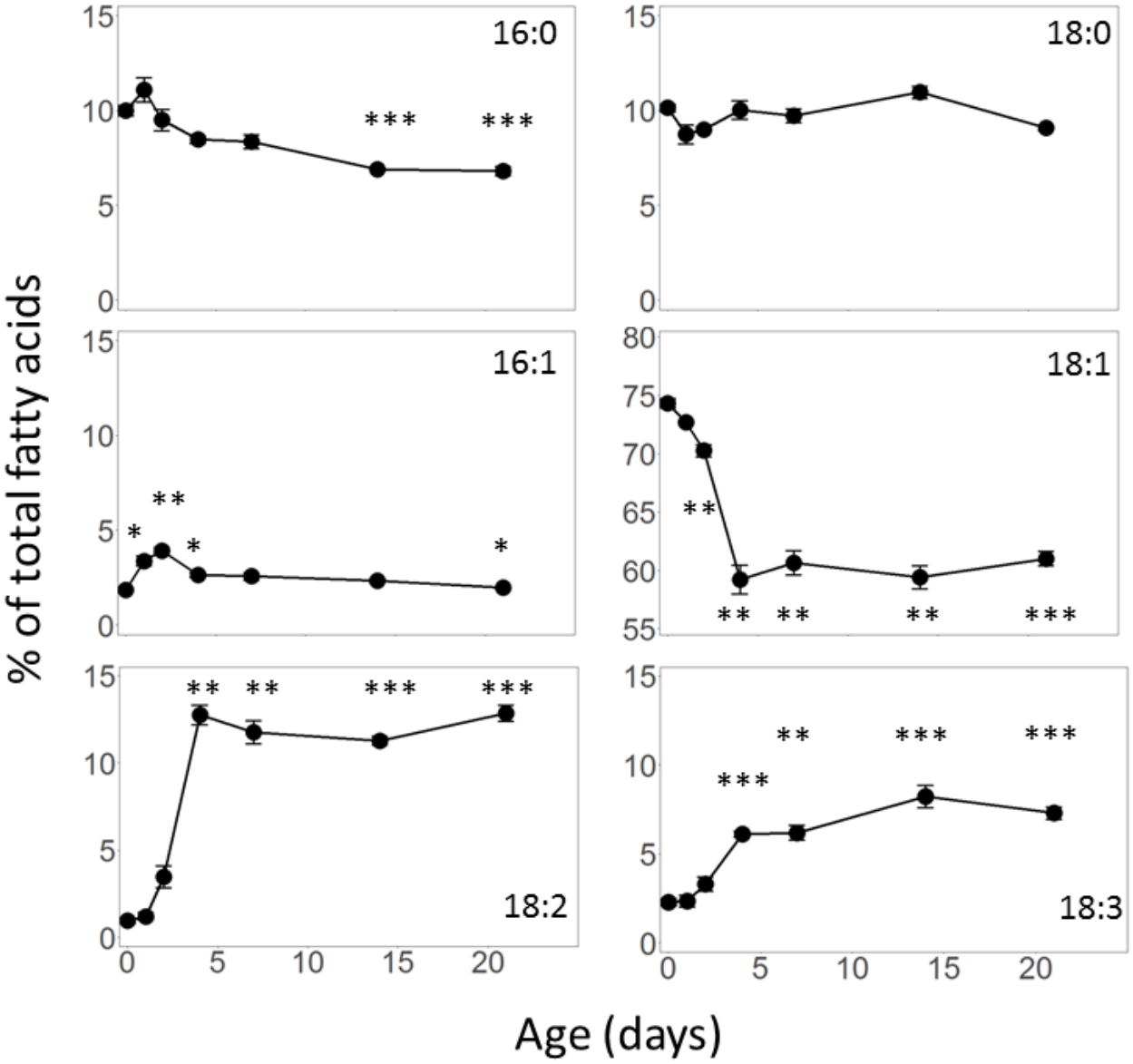


Figure 3. 4. Fatty acids characterised in whole individual worker bee membrane phospholipids at different ages. Ages characterised were emergent (0), 1, 2, 4, 7, 14 and 21 days. Data are presented as mean ± s.e.m. and expressed as percent (%) of total fatty acids. Where error bars are not obvious, error bars are less than the size of the marker. $N = 3$ for each age. * $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$ indicate the level of significant difference from emergent worker bees.

### 3.3.5 Peroxidation index

The peroxidation index (PI) of bee membranes remained low (~5) during the first 24 hours post-emergence in adult worker bees (Figure 3.5) but by the second day of adult life, PI had doubled (~10), and by day 4 of adult life, PI had increased 4.7-fold (value of 25) compared to emergent workers. The biggest increase in membrane peroxidation index was observed between day 2 and 4 that corresponded to the large increase in the incorporation of 18:2 and 18:3 in membranes. After four days the peroxidation index of membrane phospholipids remained at this higher level for the remainder of adult life (as measured).

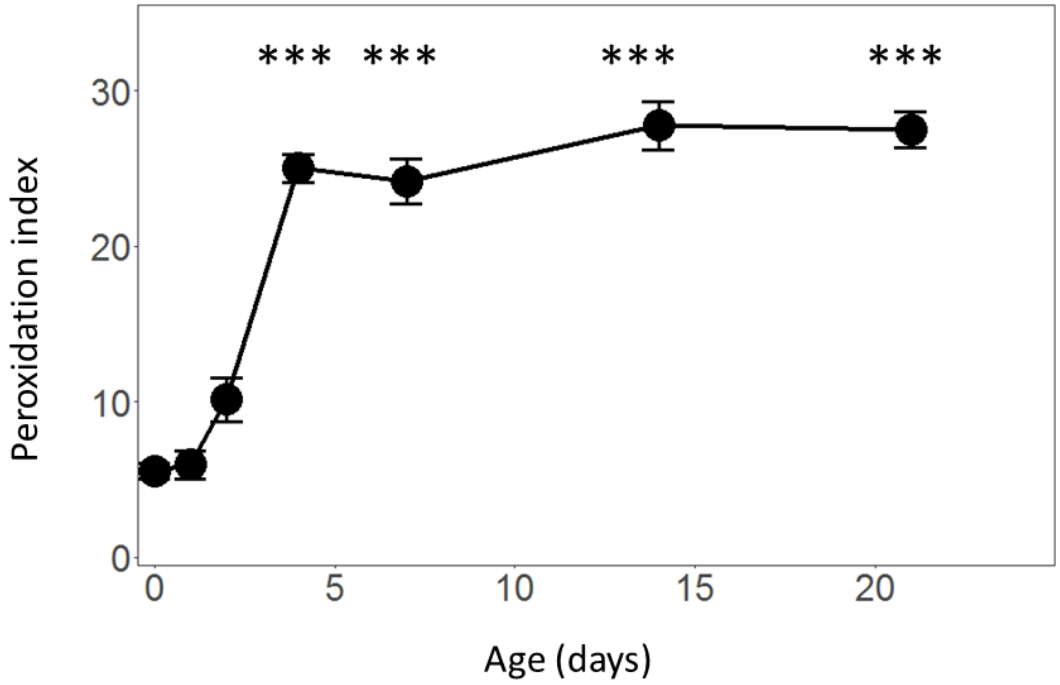


Figure 3. 5 Peroxidation index calculated from whole individual worker bee membrane phospholipids at different ages. Ages characterised were emergent (0), 1, 2, 4, 7, 14 and 21 days. Data are presented as mean ± s.e.m. Where error bars are not obvious, error bars are less than the size of the marker. $N = 3$ for each age. See section 2.2.5 for more details about the calculation of the peroxidation index. ***$p < 0.001$ indicates the level of significant difference from emergent worker bees.

## 3.4 Discussion

Previous studies have shown that changes in membrane phospholipid composition occur in adult worker bees over their lifetime (Haddad et al., 2007; Robinson et al., 1990; Chapter 2). However, no study has previously chronicled these changes over time in worker bees. By far the most notable change occurring in the fatty acid composition of worker bee membranes is the progressive increase in the proportion of polyunsaturated fatty acids (PUFA). This change is driven by two PUFA only: 18:2 and 18:3 (Figure 3.4). This relative increase in PUFA occurs over the first four days of adult life (i.e. post-emergence) primarily at the expense of the monounsaturated fatty acids (MUFA) 18:1 that decreases rapidly throughout this same period (Figure 3.4). From day 4 on, the membrane phospholipid fatty acid composition of adult worker bees stabilises with worker bees maintaining a highly monounsaturated membrane dominated by 18:1 (~60%) and with PUFA in the form of 18:2 (~12%) and 18:3 (~8%) adding significant contributions. The remaining acyl composition was mainly made up of two saturated fatty acids in the form of 16:0 and 18:0 that together constituted ~20% of total membrane fatty acids.

One of the major effects of the changes in membrane acyl composition was to increase the peroxidative capacity of bee membranes from day 1 through to day 4 (Figure 3.5). The increase in peroxidation index is caused by the increasing incorporation of the two polyunsaturated fatty acids; 18:2 and 18:3. At day 4, the peroxidation index (PI) of worker bee membranes has reached their maximum value of ~28 (compared to ~5 at emergence), a level that remained relatively stable throughout adult life. Thus, as workers begin their adult life (i.e. from emergence to day 4), their membranes become increasingly susceptible to peroxidation.

The changes in membrane composition that occur during the first four days of adult life in bees suggests that membrane remodelling is constantly occurring following emergence and continues up through to day 4 after which it stabilises. Little is known about membrane remodelling in insects. In arthropod, a massive capacity to build and dismantle membrane has been demonstrated in the outer eye segments of nocturnal spiders (Blest, 1978). It is known that deacylation and reacylation can occur extremely rapidly as demonstrated by the bacterium (*Escherichia coli*) that can replace 16:0 with 18:1 fatty acids in phospholipids within 30 seconds of cold exposure (Rock et al., 1996). It is also known from studies using

rat hepatocytes that *de novo* synthesis yields only a few phosphatidylcholine (PC) and phosphatidylethanolamine (PE) molecules (PC/ PE 16:0_18:2; 16:0_18:1; 16:0_22:6 and 18:_18:2; Schmid et al., 1995) that are then remodelled to form the dozens of molecular phospholipids present in mammalian tissues (Cortie et al., 2015; Mitchell et al., 2007). Thus, in mammals, the vast majority of phospholipid molecules are made up from the deacylation and reacylation of a few basic phospholipids.

The current results suggest that in honey bees the number of phospholipid molecules synthesised *de novo* is also very small (as found in hepatocytes of rats) but during the first day after emergence, there is a rapid increase in the abundance of phospholipid molecules (Figure 3.2; although not statistically different). Interestingly, this increase is best explained by an increase in phospholipid molecules containing only SFA and MUFA (i.e. the non-PUFA-containing phospholipids). This increase in membrane phospholipids in the first 24 hours of worker adult life suggests that workers are increasing their abundance of membranes during this period (e.g. mitochondria and other supporting membranes are required to maintain an active life after emergence). The abundance of non-PUFA-containing phospholipids then reduced progressively from day 2 of worker adult life until day 4 (to levels similar to those of emergent worker bees). However, during this unique period in the life of an adult bee, there was a compensatory increase in the level of phospholipid molecules containing PUFA. This increase appears to be delayed for the first few days of adult life when non-PUFA phospholipids are increasing then decreasing. However, phospholipid molecules associated with PUFA progressively increase from day 2 to 4 of adult life then remain relatively stable for the rest of adult life (up to three weeks).

These results suggest that worker bees first produce, by *de novo* synthesis, phospholipid molecules that primarily contain only SFA and MUFA during the first day of adult life increasing PC 18:1_18:1, PE 18:1_18:1, PC 16:0_18:1 and PE 18:0_18:1 that account for 80% of the overall change in total molecular phospholipids (Table 3.2). It is interesting that none of the molecular phospholipids of phosphatidylinositol (PIn) and phosphatidylserine (PS) contribute significantly to changes in total phospholipids at this time. After day 1, the biochemical machinery of workers starts to remodel membrane phospholipids to incorporate PUFA. This fundamental shift is likely occurring as a result of pollen consumption in the hive. The biggest increase in phospholipid molecules containing

PUFA was observed between post-emergence day 2 and day 4 (see PUFA-containing phospholipids in Table 3.2). Interestingly, the increase in phospholipid molecules containing PUFA also involves only a small number of molecules: PC 18:1_18:2, PC 18:1_18:3, PC 18:2_18:2, PC 16:0_18:2, PE 18:0_18:2 and PE 18:1_18:3 (Table 3.2). Again, phospholipids with PI and PS headgroups showed little change during this period.

The relative stability of phospholipids that contained-PUFA from day 4 onwards is associated with a general stabilisation of all PUFA-containing phospholipid molecules (as shown in Table 3.2 and Figure 3.2). The predominant phospholipid among these being PC 18:1_18:2. The stability of phospholipid composition and the proportion of phospholipids containing-PUFA means that any damage to phospholipids containing-PUFA caused by their peroxidation is effectively replaced by deacylation/reacylation mechanisms. A similar situation has been observed in rat hepatocytes where ferric stressed cells resulted in peroxidative product being formed (TBARS) but little change in the level of the major phospholipid classes (i.e. PC and PE) that contained PUFA (Girón-Calle et al., 1997). In the case of hepatocytes, there was a specific loss of PUFA from triglycerides suggesting that triglycerides are acting as a PUFA pool to replace peroxidised PUFA on phospholipids. One can only speculate on the PUFA of phospholipids of worker honey bees and their potential replacement from triglyceride stores.

### 3.4.1 Plasmalogens

Plasmalogens have been proposed as having an antioxidant capacity in membranes, acting as free radical scavengers (Engelmann, 2004). In Chapter 2, it was proposed that the higher level of plasmalogens on PUFA-containing phospholipids might aid in the longevity of the queens compared to workers. In workers, it was found that the level of plasmalogens increased consistently during their adult life. This consistent increase in plasmalogens would benefit workers with their higher levels of PUFA. However, most of the plasmalogens in workers are coupled to non-PUFA-containing phospholipids unlike those of the queens. The level of plasmalogens compared to the total level of phospholipids in worker bees is very low (~2% of total phospholipids) and thus suggests a limited capacity.

### 3.4.2 Membrane regulation with diet

The membrane phospholipid composition of honey bees does not directly reflect the

fatty acid composition of their simple diet (primarily honey (no fat) and pollen). The PUFA content of membranes in worker honey bees is ~20% of the total fatty acids whereas in pollen PUFA account for almost 50 %. Similarly, the dominant fatty acid in worker membranes is 18:1 being (~60%) whilst in pollen 18:1 only accounts for ~5% of total fatty acids. However, although there is no direct correlation between diet and membrane composition, there is certainly an influence of diet as shown in the case of membrane PUFA and pollen. In a review of 31 different pollens (mainly from Western Australia), it was found that the total PUFA content of pollen averages around 46.9 ± 1.8 % (mean ± SEM, $n$ = 31) of total fatty acids (Manning, 2006). Thus, the large increase of PUFA in membrane phospholipids found in the current study after their emergence as adult bees and their consumption of pollen is indicative of pollen PUFA influencing membrane composition during adult life of workers. Pollen in the form of 'bee-bread' has a similar level of 18:2 and 18:3 (25 and 23%, respectively, Table 3.1). However, the membrane phospholipids of adult workers have a slightly higher proportion of 18:2 compared to 18:3 (13 versus 8%, respectively), suggesting that 18:2 is preferably incorporated into membrane phospholipids. The incorporation of a higher proportion of 18:2 instead of the 18:3 would lead to a lower membrane peroxidation index.

Membrane fatty acid regulation has been reported in some animal models. For example, in rats, the membrane phospholipids of several organs (brain, heart, skeletal muscle, liver, kidney) and blood (plasma and red blood cells) are strongly regulated in respect to diet (Abbott et al., 2010, 2012). Diets that varied only in their dietary fat types (from 1 to 80% in PUFA content) caused no change in the relative proportion of total PUFA in membrane phospholipids. In these experiments, the composition of membrane phospholipids was more responsive to the n-3/n-6 balance than to the total PUFA content of the diet. Rainbow trout mitochondria have also shown similar regulation of their membrane phospholipids when fed high PUFA diets (Martin et al., 2013). Mammals and freshwater fish (such as rainbow trout) can use *de novo* enzymes to elongate and desaturate PUFA acquired from the diet before incorporating them into their membrane phospholipids or triglycerides. In contrast, bees lack the ability to elongate and then further desaturate precursor n-3 and n-6 PUFA to make longer PUFA chains. Therefore, in honey bees, the selection of PUFA that can be incorporated into membrane phospholipids is presumably limited to those obtained directly from the diet.

Interestingly, membrane peroxidation index (PI) has been found to be regulated and maintained at constant levels in membrane phospholipids in all organs and blood fractions in rats (Hulbert et al., 2014). For example, in the previous studies mentioned (Abbott et al., 2010, 2012) the PI of the different fat diets varied between 1-150 yet produced marginal changes in the PI of tissue membranes between 7-18%. This response suggests that animals regulate their membrane phospholipids to maintain a relatively set peroxidation capacity. A similar situation was observed in blowflies (*Calliphora stygia*) were diets of varying fat composition showed limited ability to change membrane fatty acid composition, with larva and adult workers maintaining similar low membrane PI values regardless of their diet (Kelly et al., 2014). Blowflies, like honey bees, are unable to elongate and desaturate dietary fatty acids. The results of all these experimental investigations suggest that membranes maintain a set peroxidation index. In worker honey bees, it appears that membrane PI is set at day 4 and then maintained throughout adult life (monitored up to three weeks in the current experiment).

## 3.5 Conclusion

Worker honey bees transit through a series of behavioral life-history stages during their relatively short lifespan. During their larval stage workers are fed mainly on royal jelly by adult worker bees, similar to queens and drones. However, once they emerge as adult, workers start consuming pollen. This change of diet leads to a progressive change in their membrane phospholipids. Surprisingly, this change is fairly fast as worker membrane composition appears relatively stable after four days of adult life. Detailed characterisation of worker membranes show that during the first 48 hours worker bees increase their amount of total phospholipid and this change is best explained by an increase in phospholipid molecules that contain only saturated (SFA) and monounsaturated (MUFA) fatty acids. After 48 hours, the level of molecular phospholipids associated with polyunsaturated fatty acids (PUFA) starts to increase progressively. Workers reach their maximum level of PUFA associated phospholipids by day 4 of adult life where it remains for the duration of their adult life. It is postulated that these changes in the membrane phospholipids of workers are explained by the modification of pre-existing phospholipid molecules. Accordingly, phospholipid molecules associated with SFA and MUFA are produced *de novo,* early on during life. After the second day, workers start to gradually modify these existing phospholipid molecules by replacing

SFA or MUFA with a PUFA acquired from pollen consumption. This process appears to involve a limited number of phospholipid molecules. This membrane remodelling also occurs during the first four days of adult life and remains relatively stable thereafter.

In Chapter 2, we have reported that workers and queens differ in their membrane phospholipids as adults and this difference was hypothesised as a mechanism that could explain the difference in lifespan between the long-lived queens and short-lived workers. The current chapter has expanded on the initial findings and found that most of the changes in worker membrane phospholipids happen during the first four days of adult life. The following chapter will test the idea of the influence of membrane susceptibility to peroxidation on longevity, by eliciting changes in the membrane phospholipid composition of adult worker bees while measuring its effect on lifespan.

# PART B. Influence of membrane phospholipids on the lifespan of worker honey bees (*Apis mellifera*) in captivity.

# Chapter 4. Diet fatty acid profile, membrane composition, and lifespan: an experimental test of the pacemaker theory of ageing using adult worker honey bees (*Apis mellifera*).

# 4.1 Introduction

The results described in the previous two chapters show how female adult honey bees (queens and workers) differ from one another in their membrane phospholipid compositions. However, the membrane phospholipids of larvae and pupae are similar between the genetically identical long-lived queens and short-lived workers. These differences in membrane lipid composition during adult life can be attributed to differences in nutrition. During larval stages, both female castes are fed royal jelly and accordingly their membrane phospholipids are very similar up to emergence. However, following emergence, worker bees start to feed on pollen in the form of 'bee bread' whilst queens continue to feed exclusively on royal jelly. Pollen has a high proportion of polyunsaturated fatty acids (PUFA) while royal jelly has a negligible level of PUFA. Consequently, the membrane lipid composition of adult worker bees increases rapidly in its level of PUFA following emergence, reaching a maximum level of PUFA after four days of adult life (see Chapter 3). After this period, the level of PUFA in worker membranes remains high and constant for the rest of their adult life. In contrast, the membrane phospholipids of queens remain highly monounsaturated, with a low level of polyunsaturated fat throughout their adult life. This distinct difference in membrane composition between workers and queens suggests that queens avoid feeding on pollen their whole life.

The increased presence of PUFA in the membrane phospholipids of worker bees means that their membranes are increasingly susceptible to peroxidation (i.e. as indicated by a high membrane peroxidation index value) compared to the peroxidation resistant membranes of queens (i.e. low membrane peroxidation index value). The difference in membrane peroxidation index values between the different membranes of the two female castes has been proposed as a mechanism to explain the difference in longevity between queen and worker bees (Haddad et al., 2007; Chapter 2). This question seems highly testable based on the fact that differences in the diet appear to be the main reason for the two sets of membranes being different in their composition and peroxidative index. It begs the question, could workers live for longer if the peroxidative index of their membranes was maintained at a low level post-emergence? To test this hypothesis, groups of emergent worker bees (with low membrane PI values) were kept in enclosures that mimic conditions in free-living hive but were fed separate diets that differed in their fatty acid composition, and their lifespan was

monitored. Four treatment regimes were used for these experiments; i) normal pollen (that contained PUFA and a variety of proteins), ii) a yeast based diet (a diet with a variety of proteins but NO PUFA) and, iii) a casein (single protein) based diet with or without added PUFA (from vegetable oils). All diets were matched for protein content and calorie content.

Trials were used to establish optimal conditions for animal housing and also to demonstrate that worker membrane phospholipids could be altered in a predictable fashion (Figure 4.1).

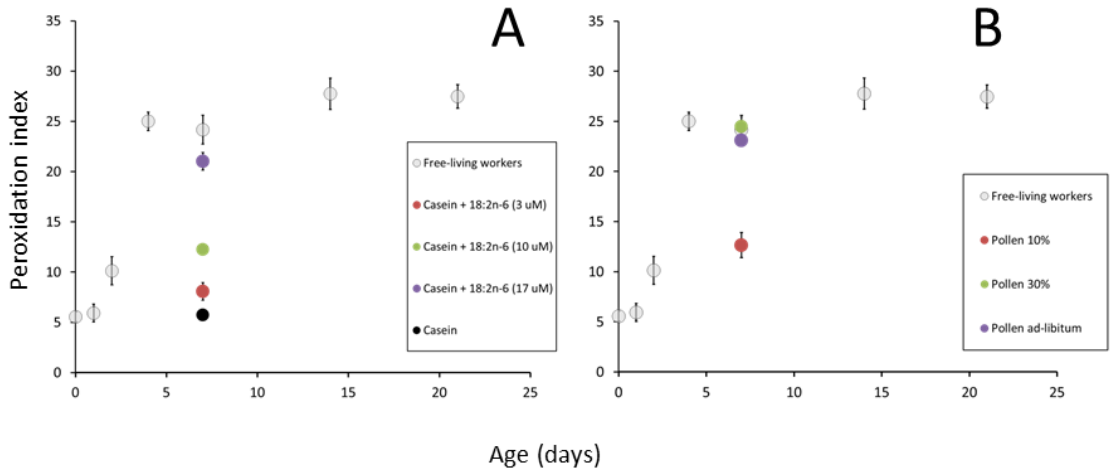


Figure 4. 1. Membrane peroxidation index of worker bees fed different diets. A) Emergent workers were fed various doses (0, 3, 10 and 17 uM) of pure linoleic fatty acid (18:2n-6) in a casein: honey solution (prepared as described in section 4.2.3). B) Emergent workers fed various concentrations of pollen mixed in honey solution. *Ad-libitum* treatment was provided using a frame with pollen and honey taken from a free-living hive. Data are presented as mean ± s.e.m., and all bees were sampled at seven days post-emergence ($n$ = 5). Both trials show a stepwise increase in membrane peroxidation index with an increase of either linoleic pure fatty acid dose or pollen concentration (%) in the diet. Light grey markers represent membrane peroxidation index values from free-living workers (at different ages from emergence to 21 days, see Chapter 3 for details). These trials were used to optimize the diets. Preliminary results also showed that membrane peroxidation index of worker bees maintained in cages increased progressively from emergence to 7 days (data not shown). The trials also showed that the addition of pure fatty acids in food was associated with high mortality rates (data not shown) in worker bees (potentially due to the toxicity of free fatty acids) and fatty acids were therefore provided from a triglyceride source (vegetable oils).

## 4.2 Material and Methods

### 4.2.1 Cages

Each treatment group was housed in a cage structure that provided a mini-hive (30 cm height, 40 cm wide 10 cm long) coupled to a netted enclosure (30 cm X 30 cm X 30 cm). The mini-hive was made of perspex with dimensions fitting a single frame in a normal hive. At the beginning of the experiment, each cage was supplied with a single brand new frame of foundation wax. The netted enclosure was made of lightweight aluminium covered in mosquito netting. The top section of the cage was fitted with a modified mosquito netting that allowed full access to the cage and hive to perform tasks such as cleaning, replacing food and collecting dead bees. One side of the hive was fully open to the netted enclosure (Figure 4.2). The cages were maintained in a greenhouse tent system (HB Commerce Pty Ltd, NSW, Australia) and set-up on shelves in a temperature-controlled room (Figure 4.3).

### 4.2.2 Temperature and relative humidity

The relative humidity of all cages was controlled via a customised made system connected to two humidifiers (Ultrasonic humidifier, CA, USA). Cages were kept at a calibrated relative humidity of 60.0 ± 0.5 %. Eight (8) mini-fans (Homebrand, NSW, Australia) provided constant ventilation within the greenhouse enclosure (see Figure 4.3). The temperature was maintained at 31.0 ± 0.3°C (mean ± s.e.m.) with temperature and relative humidity monitored every 10 min using data loggers (Hasta Data Loggers, NSW, Australia) located on the top of each cage.

### 4.2.3 Diet preparation

Honey was purchased from a commercial supplier (Natural View Apiary, NSW, Australia) and maintain at 4°C. On the day of food preparation, honey was pasteurized at 65°C for 30 minutes and water (Milli-Q®) added at 25 % (w/w) to decrease the viscosity of the honey. This ratio (honey: water of 3:1) was used for all diet preparations. Honey and water were mixed at 65°C until fully blended. Pollen was purchased from Australia's Own Pty Ltd (NSW, Australia) and classify as Jarrah Gold pollen. Rennet casein was purchased from Rogers & Co Edible Rennet, Queensland, Australia.

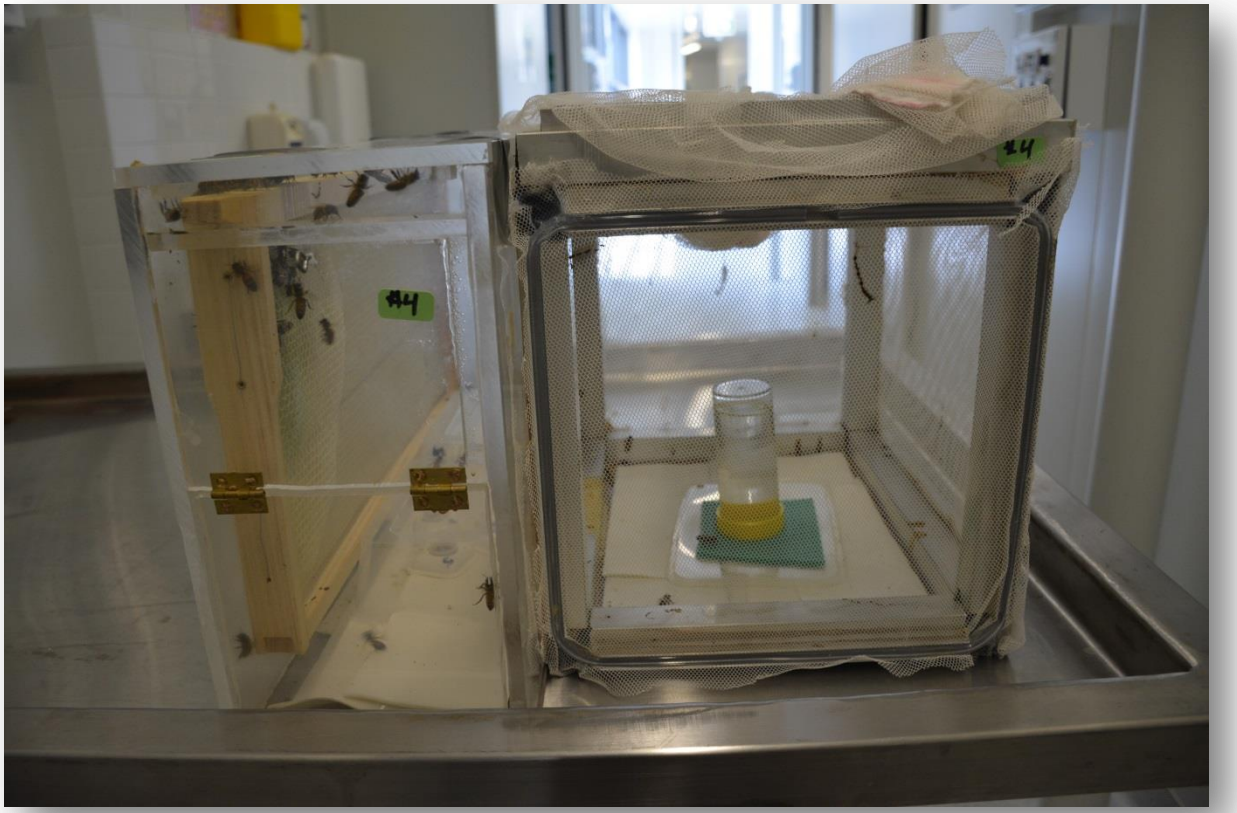

Figure 4. 2. An example of one of the cages used as a mini-hives to maintain worker honey bees (*Apis mellifera*). Each cage had a 'hive area' that could fit a regular frame and where the food was provided (left side). The mini-hive was connected to a cage where workers could fly to defecate (right side; see section 4.2.1 for details).

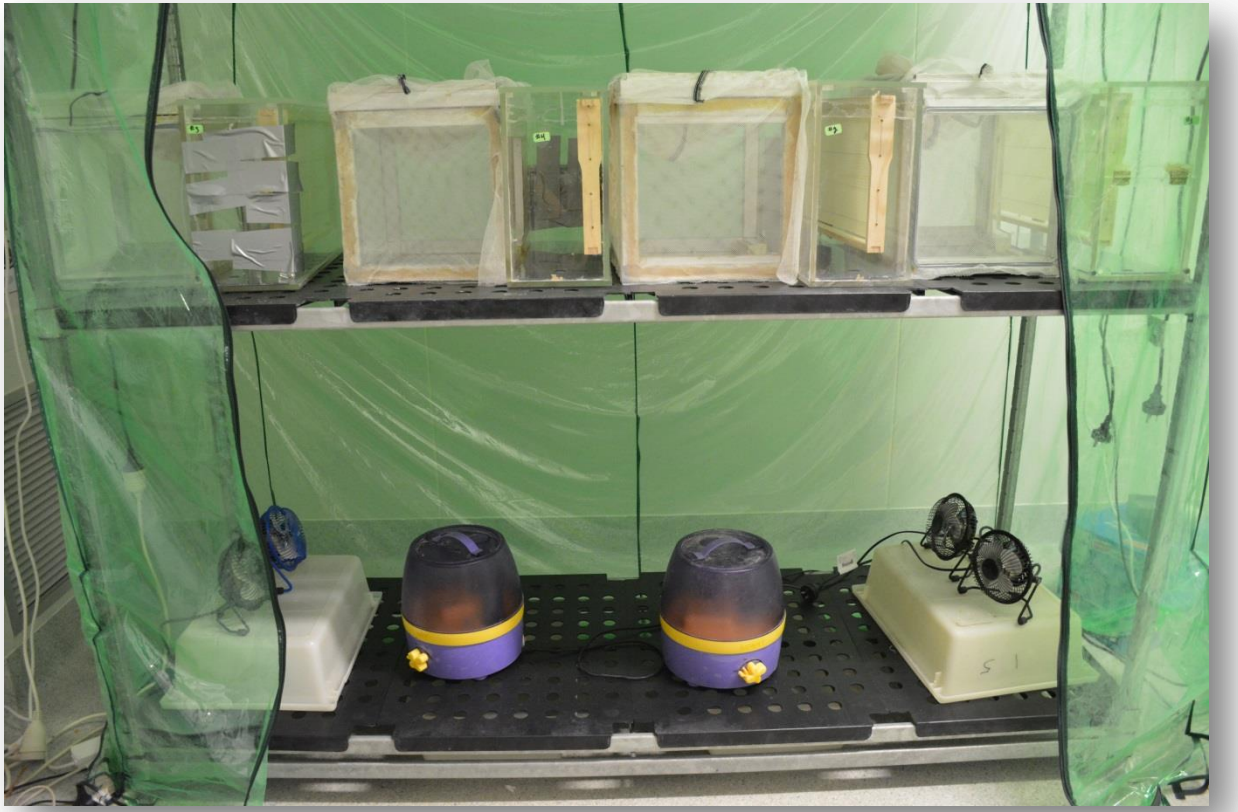

Figure 4. 3. Temperature controlled room used to maintain worker honey bees (*Apis mellifera*). Perspex hives were maintained in a greenhouse within a temperature controlled room at 31.0 ± 0.3°C (mean ± s.e.m.). Relative humidity was controlled via a customized system connected to two humidifiers and calibrated at 60.0 ± 0.5 %. Mini-fans (four at the bottom and four at the top of the greenhouse, not shown) provided constant ventilation within the greenhouse enclosure.

Yeast was obtained from Lallemand wine yeast (South Australia, Australia). Small amounts of each protein (casein, yeast or pollen) were added to the honey water mixture under constant mixing to facilitate homogenisation of each ingredient into the honey: water solution until the desired protein concentration was reached. The resulting solutions were mixed for a further 15 minutes at 65°C to ensure a homogeneous solution. Aliquots (20 mL) aliquots of each food solution (pollen, yeast, casein, and casein + PUFA; see below for details on PUFA addition) were taken and immediately chilled and stored under nitrogen atmosphere at -80°C. Aliquots of food were taken for fatty acid analysis with C23:0 fatty acid (100 *u*g) an internal standard added. The same protocol was performed for all forms of food used, including the diets with added fat; described below.

### 4.2.4 Use of vegetable oils in the diet

Flaxseed oil (Melrose Laboratories Pty Ltd, NSW, Australia) and safflower oil (Melrose Laboratories Pty Ltd) were blended as a source of triglycerides to achieve the desired fatty acid composition in the casein plus PUFA diet (Casein + PUFA). Soy lecithin (Melrose Laboratories Pty Ltd) was used (0.5% w/w) as an emulsifier to dissolve the vegetable oils into the food mixture. The desired volume of safflower and flaxseed oils were added to the soy lecithin and left under a nitrogen atmosphere for 45 minutes to allow soy lecithin to fully absorb the vegetable oils. After 45 minutes, water (at 23°C) was added to the soy lecithin and the food mixture (soy lecithin + vegetable oils + water) was maintained under nitrogen at room temperature (23°C) for a further 60 minutes, to allow the soy lecithin to fully dissolve in water. After 60 minutes, the fat solution was gradually added to the honey at 65°C until the desired concentration was achieved. The food mixture (soy lecithin, vegetable oils, water, and honey) was left for a further 30 minutes to ensure full homogenisation of the soy lecithin in the food mixture under a nitrogen atmosphere. After 30 minutes, small amounts of casein were added to the food mixture until the desired concentration was reached, with a further 15 minutes to insure full homogenisation of all ingredients (under nitrogen). Finally, 20 mL aliquots of the food mixture were taken and stored under a nitrogen atmosphere at -80°C. Preliminary experiments were used to establish the optimal amounts of vegetable oils and soy lecithin needed to achieve the desired fatty acid composition of the diet.

### 4.2.5 Fatty acid composition of diets

Fatty acid composition of each diet was determined using gas chromatography (GC). Food aliquots (500 *uL*) were weighted (±0.01 mg) into glass vials, mixed with 10 volume of methanol (MeOH):tertiary butyl methyl ether (MTBE;1:2 (v/v)), 300 µL of 150mM of ammonium acetate, 100$\mu$g of C23:0 internal standards with 0.01% (w/v) butylated hydroxytoluene (BHT) added. Vials were capped under nitrogen and gently inverted for 24 hours. Vials were then spun at 2,000*g* for 10 minutes, and the lipid fraction collected, evaporated off under nitrogen before being transmethylated and fatty acid analysis conducted as previously outlined in section 3.2.5.

The final fatty acid compositions (and fat content) of the four diets used in the experiments is shown in Table 4.1. The casein diet was devoid of fatty acids, as expected, whereas casein+PUFA showed similar levels of total PUFA (69 versus 61%), 18:2 (60 versus 50%), 18:3n-3 (10.4 versus 9.7%) and 18:3n-6 (2.8 versus 1.6%) compared to pollen. The peroxidation index of these two diets was similar at 80 versus 73 respectively. Differences between the two diets included 16:0 that was lower in casein+PUFA (14 versus 27%) and 18:1 that was slightly higher than in the pollen diet (12 versus 8%). In contrast, the yeast diet lacked PUFA instead containing predominantly monounsaturated (18:1 + 16:1 = 67%) and saturated (16:0 + 18:0 = 33%) fatty acids. Thus, four different fat diets were created; two diets with a high PUFA content either as a natural (i.e. pollen) or synthetic (i.e. casein+PUFA) fat mixture, one highly monounsaturated fat diet (yeast) and one diet with no fat present (casein).

### 4.2.6 Protein content and calorie contents of the diets

Protein content was measured by a Bradfort assay (Bradford, 1976). Protein content was the same in all diets (average 0.07 mg protein$\cdot$ mg$^{-1}$ of food). The protein content of the four diets is reported in Table 4.1. The calorie content of the diets varies between 1272 to 1380 mj. 100 mg$^{-1}$.

Table 4. 1. Fatty acid composition, protein concentration and total lipid content of the four diets.

| Fatty acids | Pollen | Yeast | Casein | Casein + PUFA |
|---|---|---|---|---|
| 16:0 | 27 ± 0.1 | 22 ± 0.1 | - | 13.9 ± 0.1 |
| 16:1 | - | 32 ± 0.5 | - | - |
| 18:0 | 2.6 ± 0.1 | 11 ± 0.2 | - | 3.8 ± 0.1 |
| 18:1 | 8.0 ± 0.2 | 35 ± 0.4 | - | 12 ± 0.4 |
| 18:2 | 50 ± 0.2 | - | - | 60 ± 0.1 |
| 18:3n-3 | 9.7 ± 0.2 | - | - | 10 ± 0.1 |
| 18:3n-6 | 1.6 ± 0.1 | - | - | 2.8 ± 0.4 |
| 20:0 | 1.2 ± 0.3 | - | - | 0.3 ± 0.1 |
| Total SFA | 31 ± 0.3 | 33 ± 0.1 | - | 19 ± 0.1 |
| Total MUFA | 8.1 ± 0.2 | 67 ± 0.1 | - | 12 ± 0.1 |
| Total PUFA | 61 ± 0.4 | - | - | 69 ± 0.1 |
| Peroxidation index | 73 ± 0.3 | 0 | 0 | 80 ± 0.1 |
| Total lipid content | 19.5 ± 0.3 | 14.8 ± 0.6 | - | 14.1 ± 0.4 |
| Protein content | 0.07 ± 0.01 | 0.07 ± 0.01 | 0.07 ± 0.01 | 0.07 ± 0.01 |
| Calorie content | 1340 | 1272 | 1354 | 1380 |

Values are presented as mean ± s.e.m., and expressed as mol % for fatty acids, nmol of fatty acid. $mg^{-1}$of food for total lipid content, mg of protein. $mg^{-1}$of food for protein content and mj. 100 $mg^{-1}$ of food for calorie content, $n$ = 5 for each diet. See section 4.2.1 and 4.2.2 for details.

### 4.2.7 Measurement of food consumption

Each cage was provided with enough food dishes, to ensure food was always in excess which was replaced daily. Food consumption was determined daily by changes in weight (±0.01 mg) of the food dishes (Ohaus analytical scale, South Australia, Australia). All values were corrected for any evaporative water loss using control food dishes placed under the same temperature and humidity conditions. Daily food consumption per cage was recorded and divided by the total number of bees present in the cage that day. Food consumption is reported as the weekly average in mg of food consumed per bee per day (Figure 4.6) as well as an average amount of food consumed per bee over the whole length of the experiment (Table 4.3). Water was provided *ad libitum* using an inverted bottle sitting on a sponge.

### 4.2.8 Set-up of worker bees in cages

Fully capped brood frames (3 to 4) were left overnight in an incubator (34°C).

Emergent worker bees (< 24 hours) were tagged with a coloured marker (Artline 400$^{XF}$). Tagged worker bees were gently brushed into a cardboard cylinder and weighed in small groups (10-15 bees). Small groups of marked bees were randomly distributed to one of the four (4) diet cages. Equal amounts of bees (i.e. by mass) were distributed randomly across the four cages. These bees are referred to as helper bees. Each cage was randomly assigned to one of the dietary treatments.

Ten days after introducing the helper bees, the same amount (i.e. by weight) of newly emergent bees (following the same procedure but without marking the bees) were introduced into each cage. A minimal ratio of 1:1 (helper bees: newly emerged bees) was used to reflect conditions within a natural hive. The total number of bees in each cage had to be maintained relatively low to allow easy access for food replacement and removal of dead bees.

### 4.2.9 Membrane fatty acid composition

Membrane lipids of whole bee sampled at 10 days (after careful removal of stinger and legs) were analysed. Ten-day old worker bees were chosen to verify dietary effects on membrane phospholipid composition based on previous work showing workers maintained relatively stable membrane composition after the first week following their emergence (see Chapter 3). This was also verified in further preliminary experiments showing a progressive change in worker membrane phospholipids from emergence to 7 days and remaining relatively stable thereafter (data not shown). Total lipids were extracted as previously outlined in Section 2.2.3. Final extracts (i.e. total lipids) in MTBE were dried down using nitrogen gas. Lipid pellets were resuspended in hexane before separation of triglycerides and phospholipids by chromatography.

#### *4.2.9.1 Separation of total triglycerides and total phospholipids*

All solvents used were of high performance liquid chromatography (HPLC) grade (Sigma Aldrich, NSW, Australia) containing 0.01% w/v butylated hydroxytoluene (Sigma Aldrich, NSW, Australia). Phospholipids and triglycerides were separated from total lipids using Sep-Pak® Classic Silica Cartridge (Waters, NSW, Australia). An aliquot of the lipid extract was evaporated to dryness and lipids recovered with hexane (3 x 5 mL) and deposited at the top of Sep-Pak cartridge. Triglycerides were eluted off first with ethyl acetate (3 x 5 mL) then phospholipids were eluted with methanol (3 x 5 mL).

Solvents containing the triglyceride and phospholipid fractions were evaporated off under nitrogen. Both fractions were transmethylated for 60 min at 100°C by adding 800 µL of acetyl chloride with 100 $\mu$g of C23:0 FA (Sigma Aldrich, Sydney, Australia) added as an internal standard. Samples were analysed as previously outlined (section 3.2.5) with the fatty methyl ester containing bottom organic phase recovered. Fatty acid methyl esters (FAME) were identified and quantified using gas chromatography (Shimadzu GC-17A, NSW, Australia) and a VARIAN Fused Silica Column (50m X 0.25 mm ID, CP7419, NSW, Australia). The temperature program used for analysis was; 150°C initial temperature, 17.5 °C/min to 170 °C, 0.5°C/min to 178 °C, 15°C/min to 222°C and 2°C/min to 232°C. The split ratio was set at 25: 1 for all analyses. Individual fatty acids were identified by comparison to an external standard (FAME mix C8-C24 Sigma Aldrich, NSW, Australia) and quantified as $nmol \cdot mg^{-1}$ of bee using Shimadzu class-VP software™ (Shimadzu, 2002) and expressed as mol % of total fatty acids.

#### *4.2.9.2 Peroxidation index*

The membrane peroxidation index of whole bee lipid extracts was calculated from the total membrane phospholipid fatty acid composition data, as previously outlined (Section 2.2.5).

### 4.2.10 Statistical analysis

Membrane phospholipids of 10 day-old worker bees feeding on the different diets were compared against membrane phospholipids of emergent workers. An analysis of variance (ANOVA) with diet as factor was used for all comparisons. Groups were compared using a Tukey post-hoc test. Weekly average of food consumption was compared using an ANOVA with diet as factor. Groups were further compared with a Tukey post hoc. All analyses were performed with R software (Version 3.2.2).

### 4.2.11 Lifespan experiments

Survival curves were calculated for emergent bees i.e. those without markers. Bees that suffered accidental deaths e.g. drowning in food or being crushing during cage maintenance were excluded from the analysis. Approximately 220 bees were included in each hive for testing of each diet. Maximum lifespan thresholds were based on the last remaining

23 bees (10%) present in each diet. Day 0 corresponds to the day of introduction to the 'hive' of the newly emerged bees (i.e. at this stage helper bees were 10-day old). Over 99% of marked helper and emergent bees were recovered in each experiment. Survival curves were produced using Survival package in R software (R Core Team, 2013). Curves were compared using both COX proportion hazard ratio and Wilcoxon rank-sum test adjusted with Bonferroni using diet as the main factor. In all cases, the results from both statistical tests were the same, and only the results for the Wilcoxon rank-sum test are therefore presented. Average (all emergent bees) and maximum lifespan (define as the period for the longest living 10% of emergent bees) were analysed using non-parametric Wilcoxon rank-sum test (as the distribution of the longevity of the population were not normal). All analyses were performed using R software (Version 3.2.2).

# 4.3 Results

### 4.3.1 Membrane fatty acid composition

#### *4.3.1.1. Total phospholipid fatty acid content*

There were no significant differences in the total phospholipid fatty acid content of bees fed any of the different diets (Table 4.2). There were also no significant changes in total phospholipid fatty acid content of any dietary group compared to when they original emerged as adult bees (Table 4.2).

#### *4.3.1.2. Membrane phospholipid fatty acid composition*

The membrane phospholipid fatty acid composition of whole worker bees on the four different diets at 10 days post-emergence are shown in Table 4.2. The fatty acid classes for these groups are shown in Figure 4.4. In general, the two groups fed diets deprived of polyunsaturated fatty acids (i.e. non-PUFA; casein and yeast) shared very similar membrane phospholipid fatty acid compositions that were more similar to those of emergent workers. Differences included a relative reduction in total saturated fats levels ($p < 0.05$), primarily compensated for by a small, but insignificant, increase in total MUFA (Figure 4.4). Membrane fatty acid composition was also similar between the two groups fed the PUFA-containing diets (i.e. pollen and casein+PUFA). Both groups increased in relative total PUFA levels ($p < 0.001$), mainly compensated for by a reduction in the relative level of total MUFA ($p < 0.001$) compared to emergent workers (Figure 4.4).

The major individual fatty acids of membrane phospholipids of whole worker bees are listed in Table 4.2. Compared to the emergent worker bees, both non-PUFA diets (i.e. yeast and casein) had very similar membrane phospholipid fatty acid composition except for a ~2-fold increase in 16:1 ($p < 0.001$). The effect of dietary treatment on membrane phospholipids was also similar for both groups feeding on PUFA diets (i.e. pollen and casein+PUFA) with highly significant ($p < 0.001$) increases in 18:2 of 6.6-7.5 fold. Most of this increase in PUFA was compensated for by a relative decrease in a single MUFA 18:1 ($p < 0.001$). Interestingly, 18:3n-3 did not increase in the PUFA fed groups compared to the emergent bees even though both PUFA-containing diets possessed ~10% of fatty acids as 18:3n-3 in the diet. There was also no incorporation of 18:3n-6 in the pollen fed group even though it accounted for 1.6% of total fatty acids in the diet. In contrast, the casein+PUFA group, with slightly more 18:3n-6 in the diet at 2.8% did incorporate this fatty acid in equal part reaching 2.8% of total membrane fatty acids in the worker bees (see Tables 4.1 and 4.2). Workers feeding on casein+PUFA also increased their relative level of 16:0 compared to the original emergent worker bees.

Table 4. 2 Fatty acid composition of the total phospholipids from emergent worker bees and at 10 days of age after feeding on the four fed different diets.

| **Fatty acids** | **Emergent** | **Pollen** | **Yeast** | **Casein** | **Casein + PUFA** |
|---|---|---|---|---|---|
| 16:0 | 7.60 ± 0.1 | 6.60 ± 0.3* | 5.40 ± 0.3** | 6.20 ± 0.3** | 14.3 ± 1.3*** |
| 16:1 | 4.10 ± 0.4 | 3.80 ± 0.3 | 9.30 ± 0.7*** | 8.60 ± 0.6** | 2.30 ± 0.9 |
| 18:0 | 11.1 ± 0.2 | 10.9 ± 0.3 | 8.10 ± 0.3** | 7.70 ± 0.2** | 9.30 ± 1.0 |
| 18:1 | 68.2 ± 1.3 | 50.4 ± 1.0*** | 67.6 ± 1.7 | 70.1 ± 0.8 | 40.4 ± 1.7*** |
| 18:2 | 3.30 ± 0.6 | 21.6 ± 0.7*** | 4.40 ± 1.4 | 2.70 ± 0.5 | 24.6 ± 4.1*** |
| 18:3n-3 | 4.50 ± 0.6 | 5.90 ± 0.4 | 4.50 ± 0.6 | 4.00 ± 0.8 | 4.70 ± 0.5 |
| 18:3n-6 | - | - | - | - | 2.80 ± 0.4*** |
| 20:0 | 1.40 ± 0.1 | 0.9 ± 0.1** | 0.80 ± 0.1** | 0.71 ± 0.1** | 1.70 ± 0.3 |
| Total fatty acid | 12.5 ± 1.4 | 12.6 ± 0.4 | 16.3 ± 1.0 | 13.8 ± 0.7 | 10.9 ± 1.6 |

Values are expressed as mol % for relative fatty acids, nmol of fatty acid. mg of food$^{-1}$ for total fatty acid and mean ± s.e.m., $n$= 5 for each group. Asterisk indicates significant change from emergent group with * $p < 0.05$ , ** $p < 0.01$,*** $p < 0.001$.

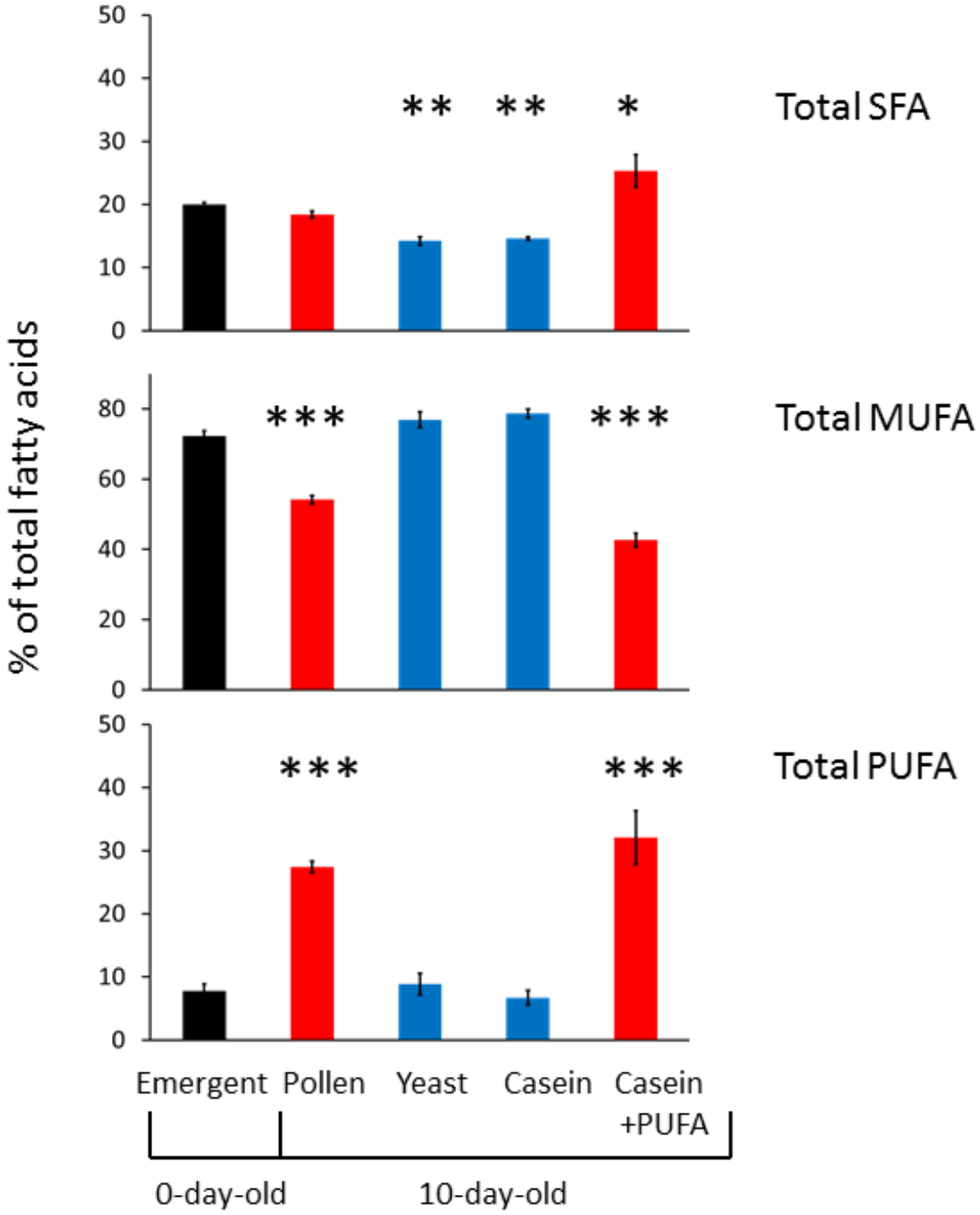


Figure 4. 4. Relative percentage of total saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids of total phospholipids from emergent worker bees and at 10 days post-emergence after feeding on the four different diets. Values are expressed as mol % and mean ± s.e.m., $n$ = 5 for each group. Asterisk indicates significant change from emergent group with * $p < 0.05$ , ** $p < 0.01$,*** $p < 0.001$.

#### *4.3.1.3 Membrane peroxidation index*

The peroxidation index of the membranes of the 10-day-old worker bees, fed on the non-PUFA diets (i.e. casein and yeast), showed no changes compared to emergent workers (Figure 4.5). In contrast, worker bees feeding on the PUFA-containing diets (i.e. pollen and casein+PUFA) increased their membrane peroxidation index (2.8 to 3.4 fold) compared to the emergent worker bees (Figure 4.4). The PI values of workers feeding on the PUFA-containing diets did not differ statistically ($p > 0.05$) to that of worker bees raised under free-living conditions (see Figure 2.4).

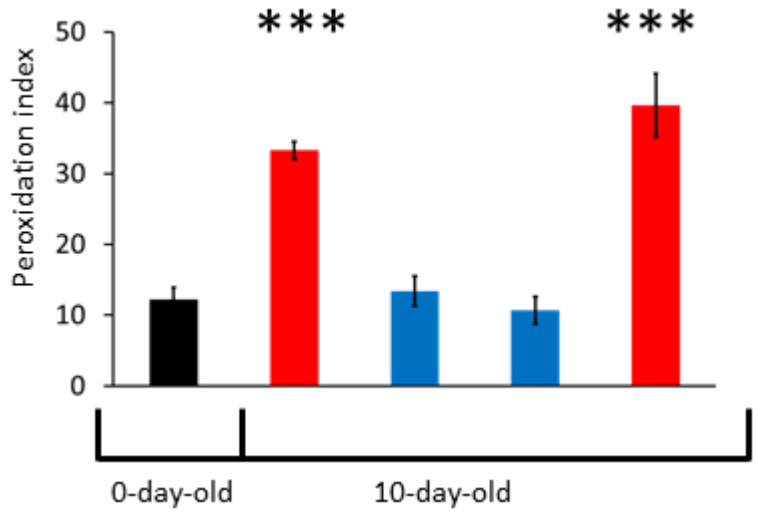


Figure 4. 5. Membrane peroxidation index of emergent worker bees and at 10 days of age after feeding on the four different diets. Details to calculate peroxidation index is provided in section 2.2.5, $n = 5$ for each group. Values are expressed mean ± s.e.m. Asterisks indicate a significant change from the emergent group with *** $p < 0.001$.

### 4.3.2 Food consumption

The average daily food consumption for each dietary treatment across the entire period of experimentation is shown in Figure 4.6, with the average daily consumption shown in Table 4.3. Both casein fed groups (i.e. casein and casein+PUFA) had a significantly higher average food consumption compared to the groups fed pollen and yeast ($p < 0.001$). The general trend for all treatments was an increase in food consumption with ageing (Figure 4.6). This age dependent increase was more pronounced for workers fed the casein based diets (Figure 4.6) with those on casein+PUFA consuming their body weight in food each day

towards the end of their lives. Worker bees feed on yeast and pollen had similar food consumption. The larger food intake recorded for worker bees feeding on both casein diets was not due to calorie content as all diets had similar calorie content (see Table 4.1).

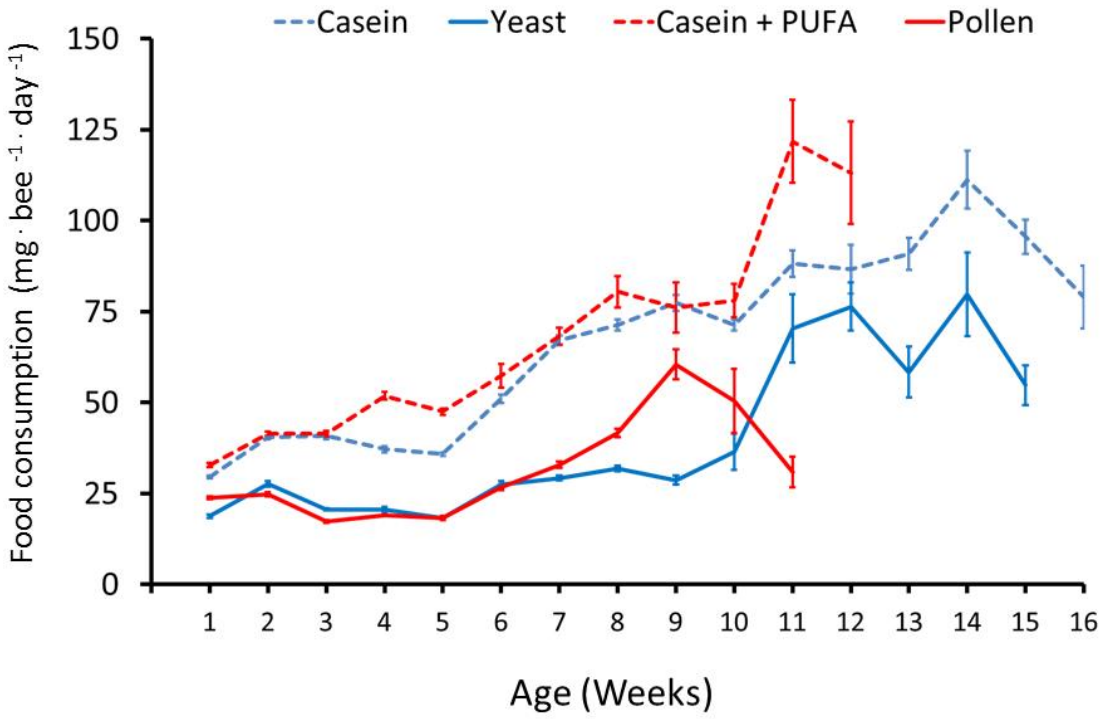


Figure 4. 6. Food consumption of adult worker bees fed on the four different diets. Data are expressed as the weekly average of mg of food consumed per bee per day and presented as mean ± s.e.m. for the whole length of the experiment. See section 4.2.3 for more details.

Table 4. 3 Average food consumption of adult worker bees (*Apis mellifera*) fed different diets.

| **Diet** | **Food consumption (mg of food. bee$^{-1}$ day$^{-1}$)** | **Food consumption (mj. bee$^{-1}$ day$^{-1}$)** |
|---|---|---|
| Pollen | 31.4 ± 4.5$^{a}$ | 426 ± 60.2$^{a}$ |
| Yeast | 39.1 ± 5.9$^{a}$ | 497 ± 75.1$^{a}$ |
| Casein | 68.4 ± 6.4$^{b}$ | 926 ± 86.7$^{b}$ |
| Casein + PUFA | 67.5 ± 7.8$^{b}$ | 932 ± 108$^{b}$ |

Data are expressed as mean ± s.e.m. Average was calculated over the entire length of the experiment. Letters indicate a statistical difference at $p < 0.001$. See section 4.3.2 for details.

### 4.3.3 Lifespan

Figures 4.7 shows the survival curves for worker bees fed pollen and yeast and Figure 4.8 shows the same curves for bees fed casein and casein+PUFA. The use of different dietary treatments, from emergence to death, had a significant effect on longevity (Log Rank Test, $\chi^2$= 25.4, df = 3, $p < 0.0001$). Yeast was used as a food source as it represents a complex ingredient (i.e. with membranes and therefore lipids, with mixed proteins, various vitamins, and minerals) similar to pollen. The second comparison, casein compared to casein+PUFA was used to more specifically examine the effect of the addition of fat (i.e. PUFA) on the lifespan of adult worker bees.

There was no significant difference between the survivorship curves of the populations fed pollen and yeast based diets (Figure 4.7, $\chi^2$= 1.6, $p = 0.129$). Worker bees fed pollen had a small but significantly higher average lifespan compared to worker bees fed the yeast diet (Table 4.4, $p < 0.01$). The average of the longest living 10% of the population (i.e. maximum lifespan) was 14% higher in workers fed the yeast diet compared to pollen (although not statistically different; $p = 0.07$). There was a significant longevity difference between the survivorship curves of the populations of worker bees fed casein and casein+PUFA (Figure 4.8, $p < 0.001$). Worker bees fed the casein diet had a significantly higher average, and maximum lifespans compared to workers fed casein+PUFA (Table 4.4; $p < 0.001$). Worker bees fed the casein diet also had the longest lifespans of all four dietary groups.

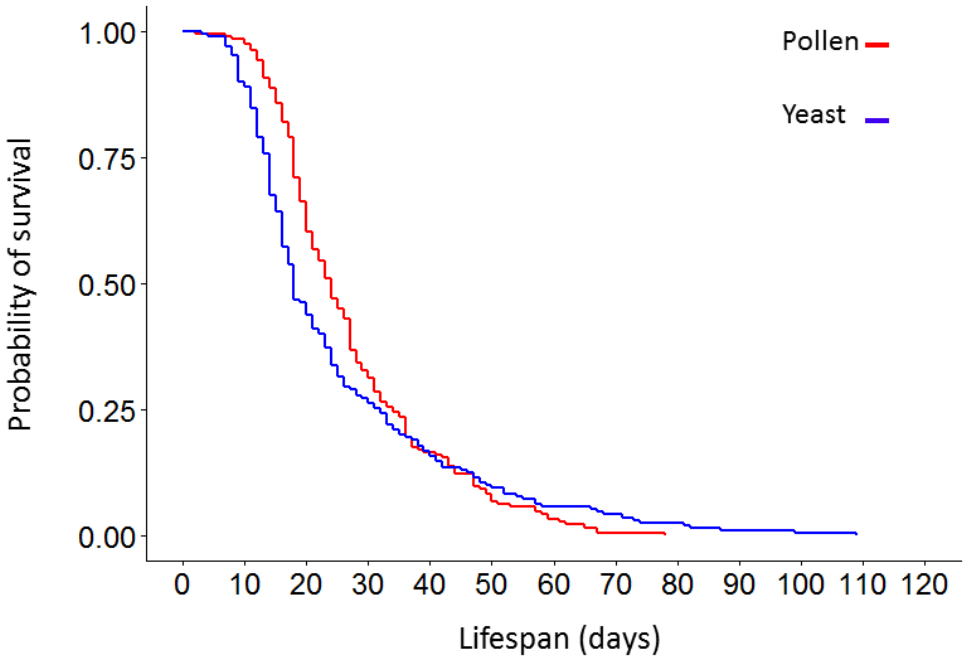


Figure 4. 7. Survivorship curves of adult worker bees (*Apis mellifera*) fed pollen and yeast diets in captivity. Survivorship was not statically different between treatments (Log rank test $\chi^2$= 1.6, d. f. = 1, $p$ = 0.21). Average and maximum lifespans, as well as number of bees used in the experiments, are provided in Tables 4.4.

Table 4. 4. Average and maximum lifespans of the four populations of bees feeding on the different diets in captivity.

| **Diet** | ***n*** | **Average lifespan (days)** | **Maximum lifespan (days)** |
|---|---|---|---|
| Pollen | 204 | $27.4 \pm 1.0^{a}$ | $56.4 \pm 1.7^{a}$ |
| Yeast | 190 | $25.2 \pm 1.3^{bc}$ | $65.4 \pm 3.6^{a}$ |
| Casein | 205 | $27.3 \pm 1.5^{ab}$ | $78.3 \pm 3.9^{b}$ |
| Casein + PUFA | 192 | $21.1 \pm 1.1^{c}$ | $56.4 \pm 2.8^{a}$ |

$N$ = 23 for maximum lifespan for each diet. Data are expressed as mean ± s.e.m. Letters indicate a statistical difference with $p \leq 0.05$ between groups, see section 4.2.11 for details. Maximum lifespan is defined as the period for the longest living 10% of the emergent population.

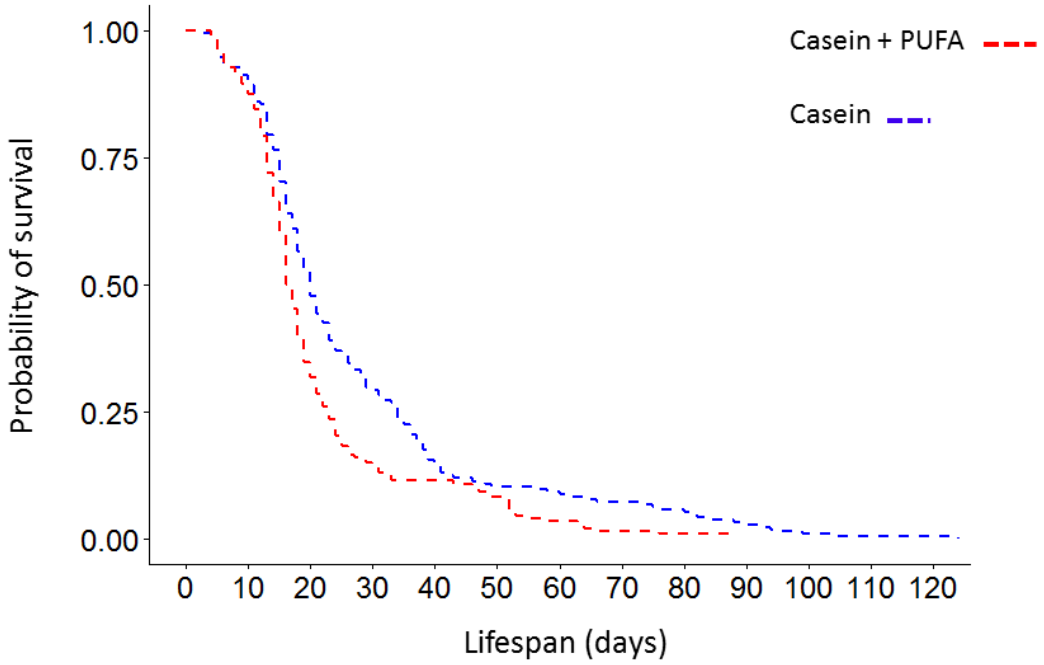


Figure 4. 8. Survivorship curves of adult worker bees (*Apis mellifera*) fed casein and casein + PUFA diets in captivity. Worker bees fed the casein diet had an increased survival rate compared to worker bees fed the casein + PUFA diet (Log rank test $\chi^2$= 13.5, d. f. = 1, $p$ < 0.001). Average and maximum lifespans, as well as the number of bees used in the experiments, are provided in Tables 4.4.

## 4.4 Discussion

The current study reports on an experiment designed to experimentally test the membrane pacemaker theory of ageing (Hulbert, 2005) using adult worker honey bees (*Apis mellifera*). The membrane pacemaker theory of ageing proposes that the polyunsaturated fatty acids (PUFA) composition of membrane lipids of a species is an important determinant of the maximum lifespan and that membrane peroxidation index is central to the biology of ageing (Hulbert, 2005). Most of the evidence supporting the membrane pacemaker theory of ageing come from correlations between the maximum lifespan of a species and their membrane fatty acid compositions (see Hulbert et al., 2007 for a review). To experimentally test the membrane pacemaker theory of ageing, it is necessary to change the peroxidation index of membranes and determine if this change affects lifespan.

Worker honey bees appear ideal candidates to test the membrane pacemaker theory of ageing as worker bees naturally change their membrane peroxidation index within the first four days of their adult life (see Chapter 3 for details). Over this unique period, worker bees, increase their membrane peroxidation index by 5-fold. It has been proposed that changes in membrane peroxidation index may contribute to the shorter lifespan of worker bees compared to genetically identical long-living queens (Haddad et al., 2007; see Chapters 2 and 3). Thus, if membrane phospholipid PUFA composition influences the lifespan of worker bees, then worker bees maintained at a lower membrane peroxidation index during adulthood should have a longer lifespan.

A major result of the current experiment was the ability of the diets tested to change the membrane peroxidation index of worker bees predictably (Figure 4.1; Figure 4.5). Both diets deprived of PUFA (i.e. yeast and casein) when fed to emergent adult worker bees resulted in worker membranes possessing a low membrane peroxidation index similar to that of the original emergent workers. In contrast, workers fed diets enriched in PUFA (pollen and casein+PUFA) increased the peroxidation index of their membranes by more than 3-fold, similar to the increase observed in free-living workers. Thus, the dietary approach used in these experiments was successful at modulating membrane peroxidation index of worker honey bees. In previous studies, it has been found that the peroxidation index of membranes is "homeostatically" regulated and therefore resistant to change. This has been found in dietary studies using rats (Abbott et al., 2010, 2012) and blowflies *C. Stygias* (Kelly et al., 2014). Essentially, the main effect of enriched PUFA diets was to increase the relative abundance of the PUFA 18:2n-6, with a corresponding decrease in MUFA, predominantly 18:1n-9 in worker membranes (Table 4.2). Surprisingly, the relative level of 18:3n-3 was similar in worker bees fed any of the diets despite being more readily available in the PUFA enriched diets (representing 10% of the total fatty acids for both pollen and casein+PUFA diets, see Tables 4.1 and 4.2). However, workers fed the casein+PUFA diet incorporated 18:3n-6 into their membrane fatty acid composition while workers fed pollen only incorporated trace levels of this fatty acid despite similar relative level in both diets (1.6 % in the pollen diet versus 2.8 % in the casein+PUFA diet). The similar changes observed in worker bee membranes fed either of the PUFA enriched diets corresponds with the similarity between the fatty acid composition of the pollen and casein+PUFA diets (Table 4.2).

These experiments provide insight and further understanding about the regulation of membrane fatty acid composition by dietary lipids as well as lipid biochemistry in adult worker bees. For instance, the experiment shows that worker bees cannot synthesise PUFA *de novo* as both groups of workers fed diets deprived in PUFA did not increase their relative level of membrane PUFA compared to emergent workers (Figure 4.4 and Table 4.2). This result is in agreement with work on *Drosophila melanogaster* that also lack the ability to synthesize PUFA *de novo* (Cripps et al., 1986). However, there are some insect species in *Homoptera* (e.g. black cheery aphid, citrus mealy bug), *Neuroptera* (e.g. green lacewing) as well as some species of the *Orthoptera* (e.g. crickets, cockroaches) that are capable of synthesizing PUFA (Cripps et al., 1986). However, bees are able to produce SFA and MUFA from non-lipid sources as shown by total phospholipid contents (Figure 4.3; Table 4.2). The relative membrane fatty acid composition between workers fed casein and yeast was very similar despite the casein diet containing virtually no trace of lipids (see details of the fatty acids of the diets in Table 4.1).

The changes in membrane fatty acid composition of bees did lead to some changes in longevity, but these changes were not consistent between comparisons (i.e. pollen versus yeast and casein versus casein+PUFA). Although the diets were designed to test for the influence of PUFA on lifespan on worker bees, another difference between the dietary treatments was the protein source. Results from the pollen versus yeast diets suggest that yeast did not have any negative effect on the longevity of the worker bees, as they achieved a similar survivorship curve compared to the natural diet of worker bees (i.e. pollen). Although the average lifespan of worker bees fed pollen was higher compared to workers fed yeast, the difference, despite being significant, was fairly small (9% increase). In the second comparison (casein versus casein+PUFA) the only difference between the treatments was the lipid content (i.e. both diets using casein as the exclusive protein source). In this comparison, the effect of increasing PUFA in worker membranes was more consistent as worker fed the enriched PUFA diet had significant lower survivorship, a significantly lower average lifespan, and a significantly lower maximum lifespan compared to worker bees fed the same casein diet without PUFA. The results from the casein versus casein+PUFA comparison are compatible with the membrane pacemaker theory of ageing. A consistent effect in both comparisons was that diets deprived in PUFA led to an extension of maximum lifespan compared to diets enriched with PUFA, regardless of the protein source (although not

significant for the yeast diet). Interestingly, both groups feeding on PUFA diet (i.e. Pollen and Casein+PUFA) had virtually the same maximum lifespan (Table 4.4). A similar result has previously been reported in worker honey bees fed diets with different concentration of linoleic acid (18:2n-6) in cages. Emergent workers fed either a diet of pollen + 16% linoleic acid or pollen + 10% linoleic acid had a significant lower longevity (i.e. survivorship) and a reduction of more than 40% in average lifespan compared to workers fed pollen only (Manning et al., 2007; Manning, 2006). Unfortunately, the experiment was stopped after 42 days even though the group fed on pollen only remained with over 50% of the population (Manning et al., 2007) and the phospholipid composition of the bees was also not measured.

The average lifespan of worker bees during summer is typically between 15-38 days in free-living conditions (Mattila and Otis, 2006; Rueppell et al., 2009; Winston et al., 1981, 1983). Workers life expectancy also varies with season (Fukuda and Sekiguchi, 1966; Winston, 1987), geographic location (Simone-Finstrom et al., 2016), population size (Rueppell et al., 2009) and genotype (Guzmán-Novoa et al., 1994; Pilne, 1980). The life expectancy of workers can drastically increase in winter as workers can survive for up to 8 months (Munch and Amdam, 2010) but the mechanism that can explain this increase in lifespan remains poorly understood. Surprisingly, and against the predictions in the literature (see Page and Peng, 2001), the average lifespan of workers maintained in cages and fed a pollen based diet is very similar to free-living worker bees. Workers maintained in cages, and fed pollen had an average lifespan between 23 to over 42 days (Manning et al., 2007; Pasquale et al., 2016; Wang et al., 2014). In all the studies referred to above, temperature (controlled between 31-34°C) and relative humidity (controlled between 50-70%) were maintained at levels similar to those found in free-living hives (Gil-Lebrero et al., 2017). In the current experiment, the average lifespan of workers fed the different diets is similar to the lifespan values above and therefore suggests that the diets and housing conditions used for the bees were appropriate to maintain adult worker bees. Unfortunately, maximum lifespan is usually not reported in most longevity studies on honey bees. However, close inspection of the survivorship curves of different studies (Hsu and Chan, 2013; Pasquale et al., 2013; Wang et al., 2014) suggests that the worker bees in the current experiment achieved similar or even longer maximum lifespan compared to most of the studies conducted in cages. The longest living individual fed pollen in the current experiment (78 days) achieved similar lifespan compared to the maximum life expectancy of workers in free-living conditions (Fukuda and

Sekiguchi, 1966). The extension of maximum lifespan does not appear to be due to calorie restriction as worker bees fed the yeast diet had similar food consumption compared to worker bees fed pollen and worker bees fed casein had similar food consumption compared to workers fed casein+PUFA (Figure 4.5, Table 4.3).

## 4.5 Conclusion

The current experiment aimed to simulate the changes observed in adult workers and experimentally test if a higher membrane peroxidation index in adult workers is responsible for their shorter lifespan compared to long-living queens. This experiment was successful in changing membrane phospholipids fatty acids of emergent adult worker bees using dietary lipids. The changes in membrane phospholipids fatty acid composition were the same regardless of the protein source used to make up the diets. Adult worker bees fed diets deprived of PUFA maintained a relatively low level of membrane PUFA and peroxidation index, similar to those of emergent workers (and adult queens), with membranes resistant to peroxidation. In contrast, adult workers fed diets enriched in PUFA increased their relative level of membrane PUFA and peroxidation index of their membranes compared to emergent workers with membranes more prone to peroxidation (like workers achieve under free-living conditions). These changes in membrane phospholipids did not lead to consistent changes in survivorship or average lifespan. However, workers fed a diet deprived in PUFA did increase their maximum lifespan by 15-30% (although not significant for the group fed yeast).

# Part C. Investigation of different physiological parameters implicated in longevity.

# Chapter 5. Measurements of ageing and metabolic rate in female honey bee castes.

## 5.1 Introduction

This chapter investigates if ageing is associated with changes in (1) ageing biomarkers, i.e. lipofuscin fluorescent pigments, and also in (2) the metabolic rates of individuals belonging to different female castes.

Lipofuscins are a group of pigments commonly used as biomarkers of ageing (Brunk and Terman, 2002; Fonseca et al., 2005; Holliday, 2006; Jacobson et al., 2010). Lipofuscins include advanced glycation (AGEs) and lipoxidation end products (ALEs) that accumulate as yellow-brown coloured granules in secondary lysosomes (Terman and Brunk, 1998). These molecules can be formed from multiple pathways (Baynes, 2000; Spiteller, 2001). For instance, protein based AGE can be formed from the autoxidation of free sugar or sugar phosphate adducts added to proteins (Maillard reaction). Lipofuscin pigments can also be formed by the autoxidation of ascorbate, as well as through glycoaldehyde adducts added to proteins from the oxidation of serine (for a review see Baynes, 2001). The pigments also contain residual products formed from the peroxidation of polyunsaturated fatty acids (Durand and Desnoyers, 1980).

Despite the complexity of lipofuscin pigments, some pigments exhibit characteristic fluorescence able to be measured using simple techniques (i.e. spectrofluorometry). Lipofuscin fluorescent pigments have been found to accumulate with age in various ageing models including yeast, *Saccharomyces cerevisiae* (Reverter-Branchat et al., 2004), the nematode *Caenorhabditis elegans* (Gerson et al., 2008) and the fruit fly, *Drosophila melanogaster* (Miquel et al., 1974; Oudes et al., 1998). Interestingly, lipofuscins are the only compounds, among several specific oxidized protein adducts, to accumulate consistently with ageing, making them good biomarkers of ageing with the potential to be predictive of the risk of death as well as predictive of cellular senescence (Jacobson et al., 2010). Since adult female honey bee castes show differences in their lifespan and level of membrane polyunsaturation (PUFA; see Chapter 2 and 3), and lipofuscins include oxidation products of PUFA, it is expected that lipofuscins will accumulate at different rates between workers and queens.

Worker honey bees transit through various life-history stages as they age, remaining inside the hive as nurses for the first part of their adult life before transitioning to the foraging

stage (see Winston, 1987 for a review on life-history stages). The different tasks performed during the different life-history stages are associated with a variety of physiological functions (Amdam and Omholt, 2003; Remolina et al., 2007; Winston, 1987). For instance, nurse bees perform tasks inside the hive such as comb-buildings, cell-cleanings, and brood care. Foragers fly distances of up to 21 km per day to collect pollen, nectar, and water to sustain and maintain the demands of the colony (Neukirch, 1982; Winston, 1987). In contrast, queens remain inside the hive for most of their life except for nuptial flights to mate with several drones (Woyke, 1962) or when swarming to establish a new colony. The main task of the queen is to lay eggs to replenish the adult worker population and insure the survival of the colony (Tautz, 2008; Winston, 1987). Since the roles of the castes differ widely, plus the fact that workers go through significant physiological changes during their transition to foraging (Amdam and Omholt, 2003), metabolic rates might be expected to differ between the female castes.

To test the hypothesis that lipofuscins accumulate at different rates in the female castes, workers and queens were sampled during pupation and at different chronological ages during adulthood (covering all life-history stages). Additionally, worker bees fed different diets in captivity were also analysed (see Chapter 4). To verify if metabolic rates differ between the castes, workers and aged-match queens were analysed in a semi-closed system that allowed measurement of their routine metabolic rate. Furthermore, the routine metabolic rate of workers fed the different diets in captivity (Chapter 4) were also measured using the same system.

# 5.2 Material and methods

### 5.2.1 Lipofuscin fluorescent pigments

#### *5.2.1.1 Source of Honey Bees and caste sampling*

Workers and queens at different life-history stages were sampled according to the methods previously described in section 2.2.2. Bees used for age pigment measurements were collected at the same time as those analysed in Chapter 2. The different life-history stages analysed for workers were: pupa, emergent, nurses (young adults) and foragers (old adults). Queen life-history stages analysed include pupa, emergent, 12-months-old (young adult) and three-years-old (old adult).

A second set of worker bees of known chronological age (method previously described in section 3.2.1) rather than life history stage were also measured for lipofuscin pigments. Collection of these bees occurred during the Austral spring of 2017. Preliminary trials showed that the paint marker on the bees did not interfere with any of the readings (data not shown). Marked worker bees were collected at emergence (0), 1, 4, 7, 14 and 28 days of age (post-emergence).

The level of fluorescent lipofuscin pigments was also measured on 10-day old worker bees feeding on the four experimental diets described in Chapter 4 (pollen, yeast, casein, and casein+PUFA). Samples were collected as described in section 2.2.2. The level of lipofuscin pigments was also measured on the longest 5% of living worker bees, for each diet. In this particular case, lipofuscin levels were measured on worker bees collected within 24 hours post-mortem.

#### *5.2.1.2 Lipofuscin fluorescent pigment measurements*

Cellular senescence/ageing was evaluated by measuring lipofuscin pigment levels were using the methods described by Jacobson et al., 2010 and Kelly et al., 2014 (Jacobson et al., 2010; Kelly et al., 2014b). Levels of fluorescent lipofuscin pigment were determined for queens and workers at different life-history stages (as described above in section 5.2.1.1). The whole bee (after removal of stinger) were homogenised in 1.36 mL of PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 1.2 mM $KH_2PO_4$ with 10 mM $Na_2$EGTA, pH 7.4) using a glass:glass tissue homogeniser with 140 μL of 10% trypsin added (i.e. 100 mg/mL of PBS + 10 mM $Na_2$EGTA, pH: 7.4). Homogenised samples were left to digest for 24 hours at 37°C before being spun at 11,000 g for 5 minutes. The resulting supernatant was removed and transferred to Eppendorf tubes with 10,000 molecular weight cut-off filters (Corning™ Costar™ Spin-X™ Centrifuge Tube filters) then re-spun at 11,000g for 5 minutes. The resulting filtrate (coloured yellow) was diluted for optimal reading on a spectroflurometer (excitation wavelength: 355 ± 10 nm, emission: 440 ± 10 nm). The dilution of the samples ensured they remained within the linear range of fluorescence. Each fluorescence measurement was corrected against blanks. Lipofuscin concentration was taken as the mean fluorescence for each sample measured in triplicate (triplicates varied by less than 5%). Lipofuscin fluorescent pigment measurements, from both castes at different life-history stages, were expressed relative to the fluorescence value measured for worker pupae

(nominally given a value of 1; i.e. each data point was divided by the mean of the worker pupa group) to facilitate comparisons among groups as previously performed (Jacobson et al., 2010; Kelly et al., 2014b). Level of fluorescence were corrected for dilutions as well as divided by the body mass (i.e. fluorescence. $mg^{-1}$) before being normalized to the worker pupae level (i.e. fluorescence. $mg^{-1}$/ mean fluorescence of worker pupa).

### 5.2.2 Metabolic rate

#### *5.2.2.1 Source of Honey Bees*

Worker bees were collected from a hive maintained at the University of Wollongong during the Austral summer of 2016. Sister queens were manually grafted according to the method described in section 2.2.2. After 14 days, queen cells were individually caged and transferred into an incubator maintained at 35°C with a relative humidity of 70 ± 5%. Emergent queens (< 6 hours) were banked into a queen-less hive and monitored for measurement. All queens were virgins when measured.

#### *5.2.2.2 Caste Sampling*

Worker bees of different age were sampled according to the methods described in section 3.2.1. Adult worker bees were collected during Austral summer of 2016 at the following ages: emergence (0), 1, 7 and 28 days of age. Worker bees of 7 days of age are referred to as nurse bees while 28-day-old worker bees are assumed to have transitioned to the foraging stage. Worker bees fed different diets (see details in Chapter 4) were also sampled at 10 days post-emergence. Finally, virgin adult sister queens were collected at 1, 7 and 28 days of age during Austral summer of 2017.

#### *5.2.2.3 Metabolic rate*

Metabolic rates were inferred from carbon dioxide production ($\dot{V}_{CO_2}$; $\mu$L $CO_2 \cdot min^{-1} \cdot mg^{-1}$of bee), using closed respirometry (Hill, 1972; Withers, 1977). Bees were placed in modified 5 mL plastic syringes with an inlet and outlet port. This chamber was chosen on the basis that it allowed to monitor body temperature and activity of bees. Atmospheric air was pulled through each syringe at a constant rate of 40 $mL \cdot min^{-1}$ STP (standard temperature and pressure), metered to within ± 1% with a Sierra Instruments mass-flow controller. Bees were maintained in the syringe till activity reached a minimum level (normally within 20-30 min).

Activity and temperature of each bee was then measured using a high definition thermographic camera (FLIR T-500 series, Victoria, Australia). Using this approach, we were able to monitor whether bees were thermoregulating or active during the measurements. Metabolic rate was defined in this study as routine metabolic rate to compare similar levels of activity by both female castes inside the hive. Bees had adequate room to move inside the chamber but were unable to fly. Chamber temperature was kept constant at 30$^{o}$C (monitored by a copper-constantan thermocouple). Once the bee settled down, both inlet and outlet ports of the syringe were closed and, the bee was kept under this closed condition for 10 min (time established using pilot runs to insure bees were not subject to hypoxia or hypercapnia). During the closed condition, the dry the atmospheric air was diverted via a bypass and monitored for $CO_2$ content (i.e. baseline). After the 10 min period, the dry atmospheric air was allowed to flow back through the syringe at a constant flow rate (i.e. 40 mL$\cdot$ min$^{-1}$).

The mixed gas (i.e., atmospheric air + gas from the chamber) was dried before entering the $CO_2$ analyzer (Sable Systems FC-1B, V. 1.01, Las Vegas, Nevada, USA). Carbon dioxide (+ 0.002%), flow rate and ambient temperature were recorded on a computer connected to an A/D converter (Sable Systems), and the signals gathered using an acquisition software (Warthog; Copyright by Mark Chappell, University of California, California, USA). The integral of the signal, obtained over time ($t$) with respect to the baseline (i.e., $f_{in}CO_2$ = 0.0003) used to calculate $CO_2$ production ($\dot{V}_{CO_2}$) was multiplied by the washout time ($\dot{n}$ = flow, in moles per time; assumed to be the same at the entrance and exit of the system), taking into account the total amount of carbon dioxide ($T_{CO2}$ in moles of carbon dioxide) present in the gas mixture ($f_{out}CO_2$) flowing into the $CO_2$ analyzer, according to the following equation:

$$T_{CO_2} = \dot{n}\left(\int f_{in_{CO_2}}\, dt - \int f_{out_{CO_2}}\,(t)dt\right)$$

The units of both integrals are % time, and the rate of carbon dioxide ($\dot{V}_{CO_2}$, in microlitres per time) was calculated by dividing $T_{CO2}$ by the time ($\tau$) elapsed when the chamber remained closed:

$$\dot{V}_{CO_2} = \frac{T_{CO_2}}{\tau}$$

Calibrations of $CO_2$ gas analyzers against known gas mixtures (4% $CO_2$ in nitrogen, and also pure nitrogen to obtain 0% $CO_2$) were performed routinely to insure accuracy. The mass-flow controller flow rate was also checked for accuracy using a chronometer and a conventional glass pipette filled with a soapy film (used as a flow meter) connected to the downstream air.

The final numbers are expressed in STPD (standard temperature and pressure dry conditions), given the fact that air going into the analyzers was always stripped of water. The corrected $\mu L$ values in STPD were calculated according to the following equation:

$$\text{Corrected } \mu L = \frac{\mu L \cdot BP \cdot 273.15\ K}{101.325\ kPa \cdot T}$$

Whereby *BP* is the barometric pressure in *kiloPascal* (*kPa*) and *T* is the temperature in *Kelvin*.

### 5.2.3 Statistical analysis

#### *5.2.3.1 Lipofuscin fluorescent pigments*

Lipofuscin levels were compared between the castes and life-history stage using an Analysis of variance (two-way ANOVA) with a Tukey post-HOC, or non-parametric Kruskal-Wallis tests, with a Wilcoxon post hoc, corrected by Bonferroni.

#### *5.2.3.2 Metabolic rate*

Metabolic rate was compared using an Analysis of variance (two-way ANOVA). Group differences were further investigated using a Tukey post hoc. All analyses were performed with R software (version 3.2.2).

# 5.3 Results

### 5.3.1 Lipofuscin fluorescent pigments

#### *5.3.1.1 Worker bees*

##### 5.3.1.1.1 Free-living worker bees

The level of lipofuscin pigments increased 4-fold from pupa to emergence in worker bees (Figure 5.1). The level of lipofuscins increased further, post-emergence, reaching its maximum level in nurse bees. Older forager bees possessed a significantly lower level of lipofuscins compared to nurse bees, with lipofuscin pigment levels more similar to those of emergent workers. The decrease of lipofuscins in foragers was unexpected. It suggests that worker bees possess the capacity to dispose of lipofuscins during their adult life. To further investigate this hypothesis, worker bees of different chronological age were sampled. These worker bees were from different locations (Wollongong, NSW versus Grenfell, NSW) and were sampled at a different time of the year (spring versus summer). However, given that the transition through different life-history stages is a major feature of the social system of honey bees, it was deemed unlikely that the location or the time of the year would influence the accumulation of lipofuscin pigments (except under severe weather conditions where bees could be confined inside the hive).

The results showed that the level of lipofuscins in both bee groups was remarkably similar and suggests that larval development leads to similar levels of lipofuscins regardless of the location and time of year. The level of lipofuscins remained fairly constant for the first day of adult life, before increasing 3.3-fold over the next three days to be at its highest point in four-day-old bees (Figure 5.1). The level of lipofuscins then decreased progressively over the next 10 days, and by four weeks, the level of lipofuscin pigments in old foragers was similar to those of emergent workers. Both groups of worker bees (Grenfell and Wollongong) analysed showed the same overall lipofuscin fluorescent pigment profile irrespective of differences in location and time of year.

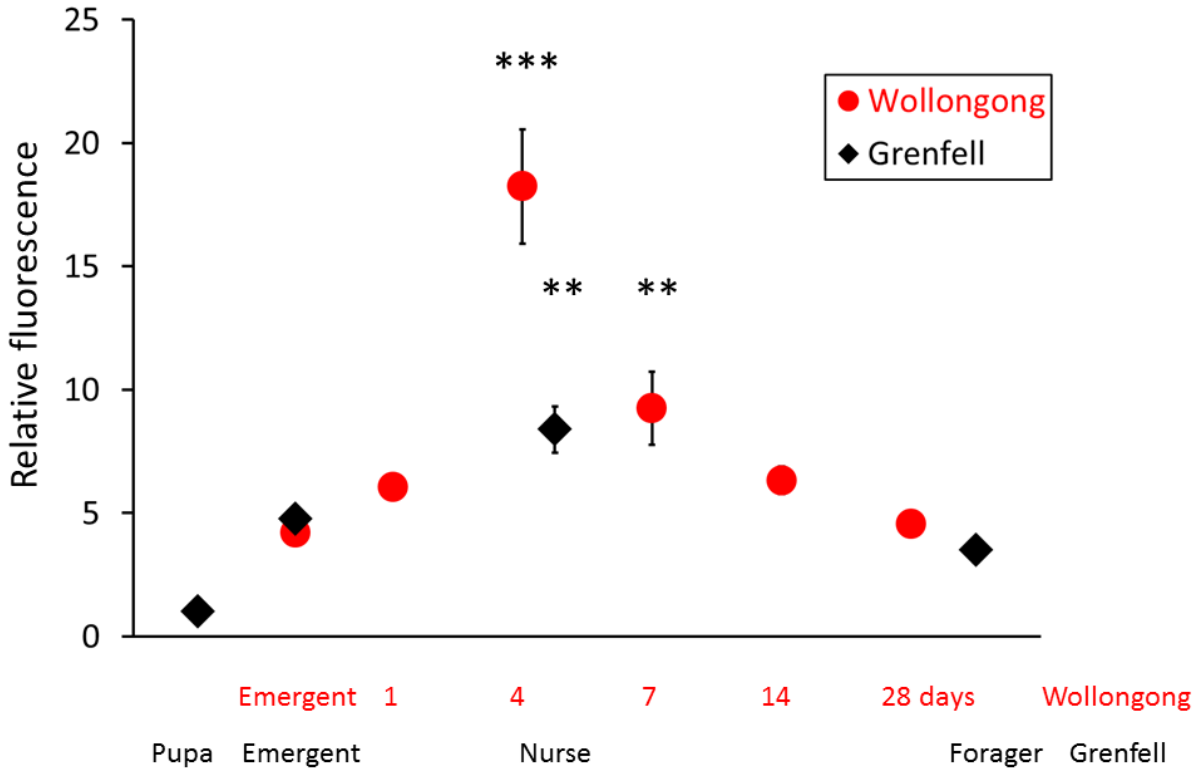


Figure 5. 1. Whole body lipofuscin pigments of worker honey bees (*Apis mellifera*) at different life-history, or chronological stages. Samples in black were collected from Grenfell, NSW, Australia while samples in red are from Wollongong, NSW, Australia. Values are expressed as mean ± s.e.m., $n = 10$. Where error bars are absent, error is less than the marker size. See section 5.2.3 for details. Asterisks indicate a significant difference from emergent adults with ** $p < 0.01$ and *** $p < 0.001$. Lipofuscin fluorescent products are expressed relative to fluorescence values measured for worker pupae.

An examination of the differences in individual variability of lipofuscin fluorescent pigment levels at the different life history, or chronological stages, is shown in Figure 5.2. This examination shows that it is unlikely that any subset of individuals, with low lipofuscins, were surviving as nurses to become foragers with low lipofuscin levels. The results also show that workers can have a great level of variability in their lipofuscins during the first week of their adult life.

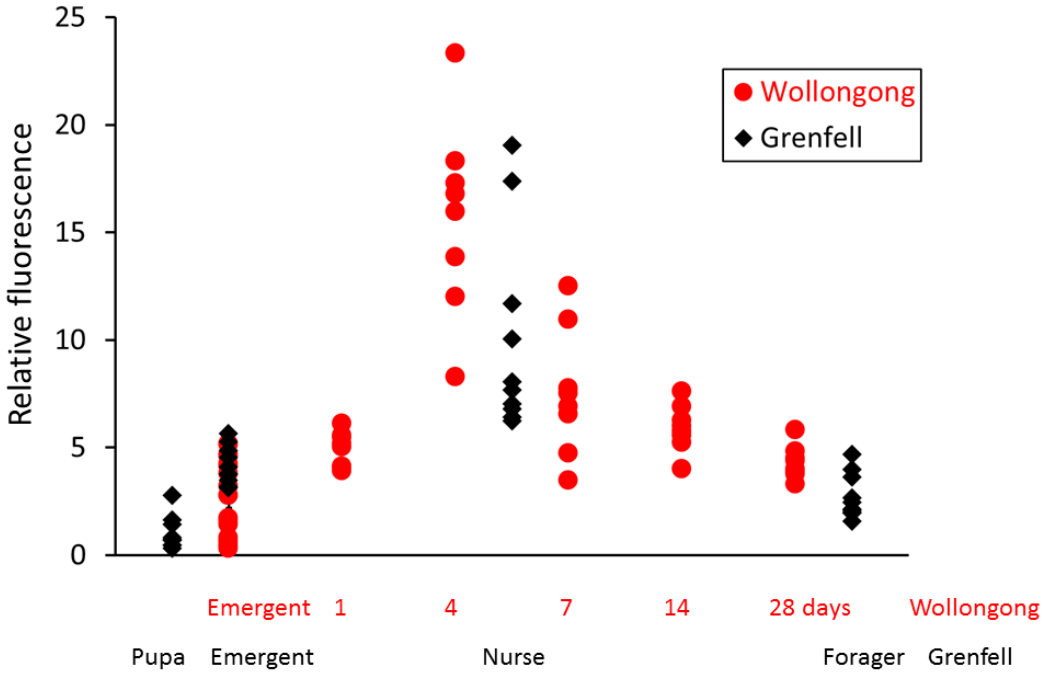


Figure 5. 2. Variance of whole body lipofuscin pigment of worker honey bees (*Apis mellifera*) at different life-history stages, or chronological stages. Each data point represents an individual. Samples in black were collected from Grenfell, NSW, Australia while samples in red are from Wollongong, NSW, Australia. See section 5.2.3 for details.

#### 5.3.1.1.2 Diets and worker bee's lipofuscin levels

The relative abundance of lipofuscin pigments in whole worker bees fed the various experimental diets (see Chapter 4) is shown in Figure 5.3. The relative abundance of lipofuscins in workers feeding on the PUFA diets (i.e. pollen and casein+PUFA) increased 2-fold in 10-day-old workers compared to the level of lipofuscins found in emergent workers ($p < 0.001$). Workers feeding on the yeast diet also increase their level of lipofuscins by 1.6-fold compared to emergent workers ($p < 0.001$). The lowest level of lipofuscins was found in the 10-day-old worker bees feeding on the casein diet, with the lipofuscin pigment level remaining similar to that found at emergence (Figure 5.3). As observed in the free-living workers, levels of lipofuscin pigments were also found to reduce with increased age. The longest living 5% of bees in all dietary treatments had low lipofuscin levels, being similar to those of emergent workers (Figure 5.3).

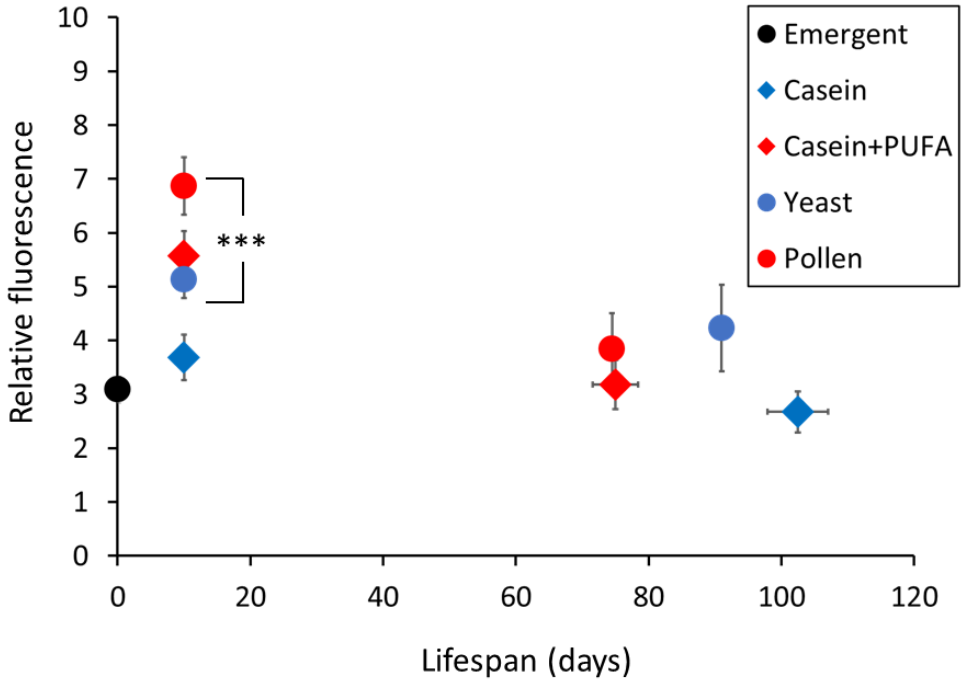


Figure 5. 3. Whole body lipofuscin pigment measurements of worker honey bees (*Apis mellifera*) fed different diets in captivity (Note: data are from experiments conducted in Chapter 4). Data on the left side of the graph are from live workers at 10 days whilst data on the right side are the longest 5% of living worker bees collected within 24 hours post-mortem. Values are expressed as mean ± s.e.m., $n = 10$ for each data point. Where error bars are absent, error is less than the marker size. See section 5.2.3 for details. Asterisks indicate a significant difference from emergent adults with *** $p < 0.001$. Lipofuscin fluorescent products are expressed relative to fluorescence values measured for worker pupae.

#### *5.3.1.2 Lipofuscins in queen bees*

The level of lipofuscins in queens is expressed relative to the average level in worker pupae to facilitate comparison between the castes. In queens, lipofuscin levels increased during development reaching the highest level as the queens emerged as adults (Figure 5.4). However, during adult life, the level of lipofuscins in queens decreased by more than 5-fold remaining low and stable in adult life (up to three years). A striking observation was that the level of lipofuscins in adult queens (12 months and three years) was only slightly higher than that of worker pupae (Figure 5.4).

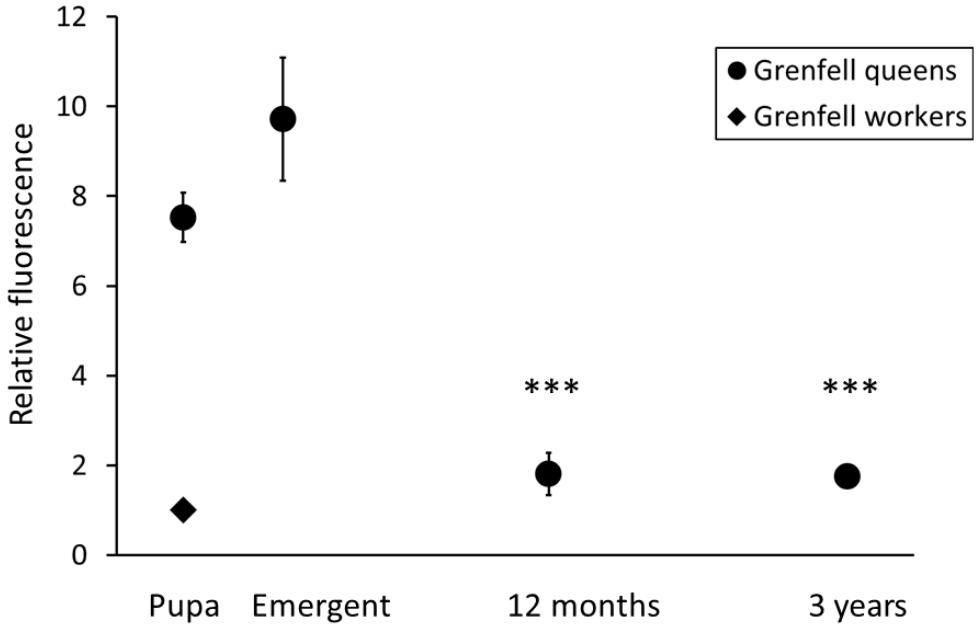


Figure 5. 4. Whole body lipofuscin pigment levels of queen honey bees (*Apis mellifera*) at different life-history, or chronological stages. Values are expressed as mean ± s.e.m. ($n$ = 8 for each stage except for emergent where $n$ = 3). Where error bars are absent, error is less than the marker size. See section 5.2.3 for details. Asterisks indicate a significant difference from emergent adult with *** $p < 0.001$. Lipofuscin fluorescent products are expressed relative to fluorescence values measured for worker pupae.

#### *5.3.1.3 Lipofuscins: queens versus workers*

Changes in the level of lipofuscin pigment between queens and workers were similar from pupa to emergence (with increases in pigment levels). Following emergence, lipofuscin pigment levels in worker bees increased several folds whereas in very young queens (i.e. 4-7 days) we currently do not know what changes occur as this was not measured in this study. However, young queens of 1 year of age were found to have low lipofuscin pigment levels as were older queens of 3 years of age. During pupation, queens had a 7.5-fold higher level of lipofuscin pigments compared to worker pupae ($p < 0.001$). Queens obtained their highest level of lipofuscins (for those ages measured) at emergence (twice that of emergent workers; $p < 0.01$) whereas workers reached their highest level of lipofuscins several days later as nurses. In adulthood, the level of lipofuscins in both queens and workers decreased

substantially, although young adult queens (12 months) maintained a significantly lower level of lipofuscin pigments compared to nurses at ~ 4-7 days of age ($p < 0.001$) and foragers at ~14-28 days of age ($p < 0.05$). Old queens (3 years of age) maintained one of the lowest low levels of lipofuscins when compared to nurse and forager bees ($p < 0.001$ and $p < 0.05$, respectively).

### 5.3.2 Metabolic rates

#### *5.3.2.1 Worker bees*

##### 5.3.2.1.1 Free-living workers

Worker bees maintained low metabolic rates during the first 24 hours of their adult life (Figure 5.5). However, over the next several days they increased their metabolic rate 5-fold ($p < 0.01$). Worker bees appeared to maintain this higher metabolic rate for most of their adult life, 28-day-old worker bees had a similar metabolic rate to seven-day-old workers.

##### 5.3.2.1.2 Workers fed experimental diets in captivity

Dietary treatments resulted in no differences in metabolic rates between the different dietary groups of adult worker bees (Figure 5.6). The average production of $CO_2$ was consistently between 0.2 to 0.3 mL of $CO_2$ produced per min per g of bee in the 10-day-old workers measured.

#### *5.3.2.2 Free-living queens*

The metabolic rate of queens of different chronological ages is presented in Figure 5.5. Queens increased their routine metabolic rate 1.6-fold during the first week of their adult life ($p < 0.01$, Figure 5.5). Queens reduced their metabolic rate slightly with ageing and 28-day-old queens had a similar metabolic rate compared to one-day old queens and seven-day-old queens.

#### *5.3.2.3 Metabolic rate: workers versus queens*

Metabolic rate was significantly higher in one-day old queens compared to one-day-old workers ($p < 0.01$, Figure 5.5). This difference between the female castes diminished with age. Seven-day and 28-day-old queens have a similar metabolic rate to those of age-matched workers ($p > 0.05$ for both comparisons).

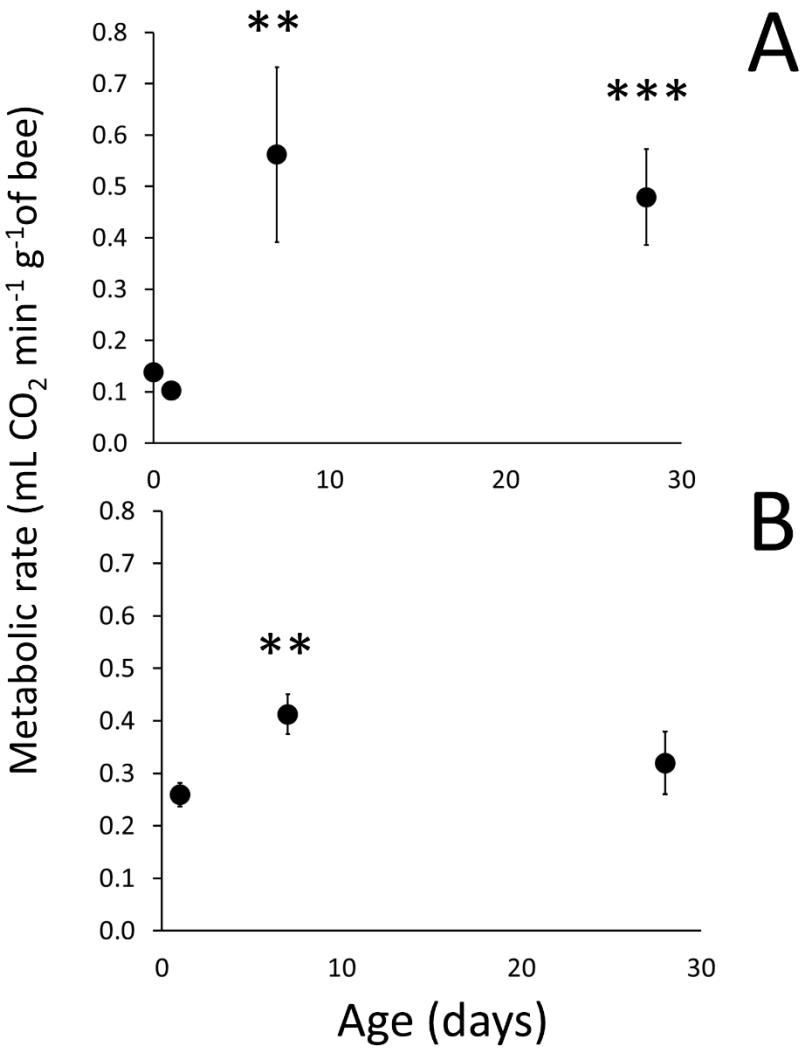


Figure 5. 5. Metabolic rates of (A) adult worker and (B) adult queen honey bees (*Apis mellifera*). Data are expressed as mean ± s.e.m. in mL of $CO_2$ per min per g of bee. $N = 7$ and above for each group, except for queens at 28 days, $n = 4$. Where error bars are absent, error is less than the marker size. Metabolic rates of female honey bees were measured at emergence (0 day; for workers only and at one day, seven days and 28 days of age for both castes, see details in section 5.2.2). Asterisks indicate that metabolic rate differed from one-day-old group, irrespective of caste (** = $p < 0.01$, *** = $p < 0.001$).

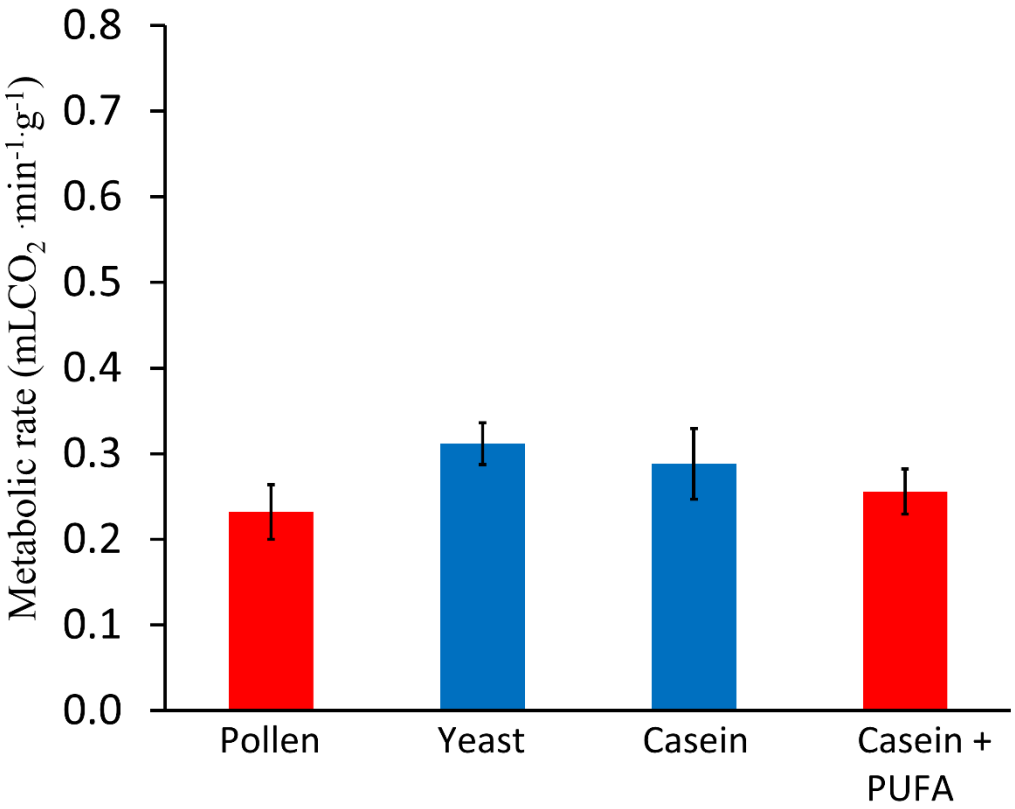


Figure 5. 6. Metabolic rates of 10-day-old worker bees (*Apis mellifera*) fed different diets. Data are expressed as mean ± s.e.m. in mL of $CO_2$ per min per g of bee. $N = 10$ and above for each group. See details in section 5.2.2 for measurements and section 4.2.3 for details on dietary treatments. Metabolic rates did not differ significantly between groups.

## 5.4 Discussion

These experiments have demonstrated the presence of fluorescent lipofuscin pigments as ageing biomarkers in the two female bee castes, i.e. queens and workers. Since lipofuscins include products of polyunsaturated fatty acid (PUFA) oxidation and the castes have different levels of PUFA in their membranes during adult life, it was hypothesised that the two female castes would differ in their lipofuscin pigment profiles. Lipofuscins were expected to accumulate to a higher level in workers, than in queens, with ageing. This hypothesis was found to be incorrect, instead of consistently increasing with age, lipofuscins initially increased but then decreased with age in both castes. Surprisingly, the level of lipofuscins in queens compared to workers was higher during pupation (7.5-fold higher) and higher at

emergence (2-fold higher). This suggests that during the early stages of development, from larva to adult form, queens produce more lipofuscins compared to workers. This difference could be an indication of a higher synthetic rate/turn-over of material as queens complete their development faster, 16-18 days versus 21 days for workers, and also grow to a larger size compared to workers (Wang et al., 2015).

As adults, the opposite trend occurred. Workers increased their level of lipofuscin pigments (queens were not measured during the earliest stages of their adult development, therefore it is not possible to comment on what changes may have occurred), with young nurse bees showing the highest level of lipofuscin pigments measured. Subsequently, in adulthood, both castes, against all expectations, decreased their level of lipofuscin pigments by 3.75-fold and 5-fold in workers and queens, respectively. The decrease of lipofuscins suggests that both castes are capable of disposing of lipofuscin pigments in adulthood. Queens were also able to maintain low levels of lipofuscins for the majority of their adult life. Three-year-old queens have the same levels of lipofuscins compared to 12-month-old queens.

Previous studies, measuring lipofuscins in insects, have only compared different stages of adult life (Gerstbrein et al., 2005; Jacobson et al., 2010; Sohal et al., 1984). However, the current study had the advantage of comparing lipofuscin accumulation at similar life-history stages covering development and adult life of the two castes. Giving the very fast development of insects (i.e. days) compared to mammals (usually in months), lipofuscins may start to accumulate significantly during development. The results of the current examination suggest that this is the case for lipofuscins in bees with substantial increases during the developmental period in both female castes (i.e. from pupae to emergence).

The biphasic change observed in lipofuscin levels in workers with ageing could be related to changes in diet, notably the ability to feed on pollen. The level of lipofuscins in worker bees raised in captivity (Figure 5.3) on diets that included pollen possessed lipofuscin levels similar to those of free-living workers (Figure 5.1). Workers fed casein+PUFA enriched diet (with PUFA content similar to that of pollen) also possessed a level of lipofuscin pigments similar to that of workers feeding on pollen. In contrast, workers fed casein diet (without PUFA) had low lipofuscin levels similar to those of emergent workers,

whereas workers fed yeast based diet (without PUFA) had an intermediate lipofuscin level. The reduction in the level of lipofuscins in workers fed a diet without PUFA suggests that the level of lipofuscins is potentially influenced by an increase in membrane PUFA content. Similar to queens, the level of lipofuscins was also found to decrease in older adult workers. This decrease was observed in two populations of free-living workers (Figure 5.1) and also observed in the longest 5% of living workers raised in captivity (Figure 5.3). The consistent reduction in lipofuscins observed in workers during adult life suggests that worker bees possess the ability to dispose of lipofuscins during their adult life. The decrease in the level of lipofuscins with ageing in both castes is intriguing as it goes against the prediction of the oxidative stress theory of ageing and other studies that show an accumulation of lipofuscins with ageing (Gerstbrein et al., 2005; Jacobson et al., 2010; Sohal et al., 1984).

In *Drosophila melanogaster,* lipofuscins have been found to increase consistently, showing a 40% increase from 7 to 40 days of age (Jacobson et al., 2010). Oudes (Oudes et al., 1998) showed that the level of lipofuscins doubled between 10 and 75 days in adult flies. Over a series of experiments, Bridges and Sohal demonstrated that both the amount of lipofuscin granules (Bridges and Sohal, 1980) and the percentage of cytoplasmic volume occupied by lipofuscin granules (Sohal et al., 1984) increased with age in houseflies. These changes were also associated with a progressive increase in pentane production (a lipid peroxidation product of n-6 fatty acids) with ageing (Sohal et al., 1985). Lipofuscins have also been shown to increase with ageing in *Caenorhabditis elegans,* tripling between 5 and 15 days (Gerstbrein et al., 2005). There are a very limited number of studies that have looked at the accumulation of lipofuscins in honey bees. One study revealed that lipofuscins accumulated progressively from emergence to three weeks in the thorax of worker honey bees (Young and Robinson, 1983). However, the levels of lipofuscins remain stable after three weeks (measured up to four weeks and also showed greater variance in older individuals compared to younger individuals, suggesting possible differences in the activity of worker bees (e.g. foraging; Young and Robinson, 1983). Lipofuscin pigments were also found to accumulate in the thorax of nurse and forager bees compared to emergent workers. However the level was similar between nurses and foragers (Young and Tappel, 1978).

The number of studies that have looked at fluorescent lipofuscin pigments in queens and drones is even more limited. One report (Young and Tappel, 1978) suggested that queens

and drones accumulate fewer lipofuscins compared to workers. Adult queens and adult drones (of unknown age) had 37% less lipofuscins in the thorax compared to adult workers (nurses and foragers). Lipofuscin granules (expressed as % of area) increased with ageing in queen (Hsieh and Hsu, 2011b) and worker trophocytes (Hsieh and Hsu, 2011c). However, a closer inspection of the results of both of these studies shows that the level of lipofuscins was relatively similar between the castes.

In only a few studies (Kelly, 2012; Martin et al., 1989) the level of lipofuscins in different sections or organelles was measured in insects. One study compared the levels of lipofuscin pigments in the thorax and brains of newly emergent and forager worker bees (Martin et al., 1989). Aging was associated with an increase in lipofuscins in the brain whereas the accumulation of lipofuscins in the thorax muscle was very limited. The level of lipofuscins was also found to be 5-fold higher in the brain compared to the thorax in forager bees. A similar result was also observed in blowfly that accumulate lipofuscins mainly in the head in contrast to thorax and abdomen (Kelly, 2012). These results suggest that lipofuscins are accumulating to a greater extent in the head (potentially more in the brain; Fonseca et al., 2005) compared to other sections of the body (i.e. thorax and abdomen) in insects. One report has shown that lipofuscin granule size increases in winter bees compared to newly emergent bees in two different regions of the brain: the calix and the pars intecerebralis (Munch et al., 2013).

The reduction of lipofuscins observed with ageing in queen and worker bees suggests that mechanisms to dispose of lipofuscins are present in female honey bees. Details about such mechanisms currently lack in the literature, and no other study so far has reported a reduction in lipofuscins in honey bees or any other social insect groups. However, previous studies on honey bees (Young and Tappel, 1978; Hsieh and Hsu, 2011b; Hsieh and Hsu, 2011c) have only look at lipofuscin accumulation in thorax instead of the whole body. Given that most of the lipofuscins accumulated in the head of insects (Kelly, 2012; Martin et al., 1989), one can argue that the accumulation in thorax might different compared to the accumulation in thorax. The disposal of lipofuscins in the honey bees appear as a novel mechanism and could be associated with their behaviour. For instance, queens spend most of their life engaged in egg-laying activities. This task is probably associated with one of the highest turnovers of biological material in the animal kingdom. In other words, a single queen

synthesises the equivalent to her body weight every few days. The high fertility and long-lifespan of queens have puzzled scientists for decades. The high synthetic turnover rates of queens associated with high reproductive investment may allow them to take advantage of incorporating some waste product (such as lipofuscin pigments) into the large number of eggs they produce. One way to test this hypothesis would be to examine eggs for the presence of lipofuscins. Given the massive level of egg production over a lifetime (as many as 8 million eggs), this may represent a good physiological trade-off for queens being able to dispose of potentially toxic lipofuscins without affecting the development of future worker bees.

Worker bees might be able to dispose of their lipofuscins by incorporating it into food secretion. Royal jelly is a mix of secretions from hypopharyngeal and mandibular glands located in the head of the nurse worker bee (Haydak, 1970). Interestingly, most of the lipofuscins appear to be located in the head in honey bees (Martin et al., 1989). Thus, one possibility for nurse bees is to dispose of their lipofuscins by incorporating some of it into their glandular secretions. In agreement with this hypothesis, workers are thought to transfer a precursor of vitellogenin to queens through royal jelly (Engels, 1974), and that could potentially explain the high level of vitellogenin in queens compared to workers (Seehuus et al., 2007). As previously mentioned, all castes are fed a liquid secretion (royal jelly) during larval stages. In contrast to workers, queen larvae are supplied throughout their larval life with an abundance of royal jelly. This difference in food allocation is correlated with a 7.5-fold difference in the level of lipofuscins between the castes at pupation (Figure 5.3). Older nurse bees also feed newly emergent worker bees with royal jelly for the first week of their adult life. Interestingly, the results from the current experiment show that the maximum level of lipofuscins for any life-history stage was found in four-day-old nurse worker bees (Figure 5.1). The largest intergroup variation was also observed in bees aged between four and seven days (Figure 5.4). This greater variation could partly reflect a difference in the amount of food ingested by young worker bees. Unfortunately, information about the amount of royal jelly produced by nurses and fed to newly emergent worker bees is scarce. The disposition of lipofuscins by incorporating it into food secretion also implies that larvae of any castes, as well as adult drones and adult queens, will also be fed royal jelly containing lipofuscins. The lower level of lipofuscins in queens during adult life suggests that the disposable mechanism in queens is very efficient, even if queens are being fed royal jelly containing lipofuscins. A partial test of this hypothesis would be to measure lipofuscins in queens after a period of cold

weather when queens stop laying eggs. This would be expected to lead to an increase in lipofuscins in queens. The reduction of lipofuscin during adult life is indeed very surprising and appear to go against the prediction of the oxidative stress theory of ageing that will predict an increase with ageing. However, the limited data on social insects suggests a complex dynamic between oxidative damage and age, which may challenge some historical tenants suggesting that oxidative damage accumulate with age. For instance, protein ubiquitination (i.e. a marker of oxidative damage to proteins) is similar between one-year-old worker ants and one-year-old queen ants, and decreased with ageing in both leg and brain tissue (Lucas et al., 2017).

The measurements of metabolic rate presented in this study represent, as far as we know, the first ones obtained from age-matched queens and workers. Since the two female castes differ in their respective roles in the hive (i.e. queen is responsible for reproduction and remains in the hive versus workers performing all the maintenance tasks for the colony, see section 1.6.1 for details), metabolic rates may differ between the two castes and could potentially explain the shorter lifespan of worker bees. The physiology of worker bees also changes as they age and move from non-flying tasks as nurses to foragers (Amdam and Omholt, 2003; Remolina et al., 2007), and could, therefore, be associated to changes in metabolic rate. The selection of age-matched workers and queens allowed for comparisons between the castes at similar ages to determine whether changes in metabolic rate occur with ageing. Overall, the experiments showed that both castes have similar metabolic rates during most of their adult life with the exception in one-day-old bees where queens had twice the metabolic rate of aged-matched workers (Figure 5.5).

Individuals from both castes increased their metabolic rate during the first week of their adult life and had similar metabolic rates at seven days post-emergence. Metabolic rates were maintained at 28 days of age in older workers while queens showed a slight decrease. The results are in agreement with previous reports that have shown that adult queens (unknown age) have similar routine metabolic rates (Fahrenholz et al., 1992), as well as maximal metabolic rates (Harrison, 1986; Harrison et al., 2005), compared to nurse and forager workers. Previous studies have also shown an increase in both routine and maximal metabolic rates in workers with ageing (Fahrenholz et al., 1992; Harrison, 1986) as it was found in the present study. Interestingly, one study revealed that routine metabolic rate in

worker bees increased rapidly between day one and day three of adult life (Fahrenholz et al., 1992). Mass-specific flight metabolic rate also increases rapidly in adult life in workers (3-4 days; Schippers et al., 2010). Flying metabolic rate increases furthermore, and the maximal rate was observed in 16 days old foragers (Schippers et al., 2010). Interestingly, the increase in metabolic rate is correlated to the same changes in citrate synthase, pyruvate kinase, hexokinase, and phosphofructokinase enzyme activities. In contrast, mitochondrial cytochrome c oxidase activity increases rapidly during the first 7 days but decreases progressively thereafter up to 25 days (Schippers et al., 2010).

The increase in routine metabolic rate observed between day one and seven in workers and queens could be related to an increase in the number of mitochondria. One report has shown that the concentration of various cytochromes (in mitochondria) increases by 10-fold within the first week of adult life in worker bees (Herold and Borei, 1963). The amount of total phospholipid in bees also increases rapidly in worker bees in the first week (Figure 3.2). These changes are likely to involve mitochondria biosynthesis with the thoracic muscles of forager workers largely dominated by mitochondria. Mitochondria occupy 43% of thorax muscle volume with a cristae surface area of 54 $m^2$ / $cm^3$ (Casey et al., 1992), a level higher to that found in mammalian tissues (Taylor et al., 1989).

The differences in longevity reported in adult workers in Chapter 4 is unlikely to be due to differences in metabolic rate as no differences in $CO_2$ production were observed between the different dietary groups (Figure 5.6). One interesting observation is that the metabolic rate of workers maintained in captivity appear to be about 40% reduced compared to free-living workers, although this difference was not significant ($p$ = 0.28).

## 5.5 Conclusion

Queens and workers accumulated lipofuscins at different rates throughout larval development. Pupa and emergent queens have higher levels of lipofuscins compared to pupa and emergent workers. This difference is reversed during adult-life with queens reducing their level of lipofuscins reduced by 5-fold, whilst workers initially increased their lipofuscin levels in the first few days of adult life followed by a progressive decrease with ageing. This reduction in lipofuscins supports the proposal that both female castes can dispose of lipofuscins with ageing. The current results also suggest that the increase of lipofuscins in

adult workers is associated with a higher membrane PUFA content. The shorter lifespan of workers may be associated with a higher amount of lipofuscins compared to the amount found in the long-living queens. However, the difference is negligible and suggests that it is unlikely to be a major explanation for the difference in lifespan between the female castes. Furthermore, the two female castes share similar metabolic rates as adults, suggesting that the rate of living hypothesis is unlikely to explain the differences in longevity between the castes.

# Chapter 6. Conclusions and perspectives.

This thesis provides the most comprehensive examination of membrane phospholipids, and their fatty acids, ever conducted in honey bees (*Apis mellifera*). It includes an examination of all castes (female workers and queens plus male drones) at six different life-history stages (larvae, pupa, emergent, and different adult stages). This thesis relates changes in membrane phospholipids to diet and behaviour of bees and also provides an experimental test of the influence of dietary lipids on the longevity of worker bees. Finally, it examines some physiological parameters implicated in longevity i.e. metabolic rate as a generator of free radicals and lipofuscins (accumulated glycation end-product of oxidative processes) that are deemed to accumulate in post-mitotic cells and therefore are indicative of ageing. The overall aim of this thesis was to use eusocial insects (in this case honey bees), with their striking difference in lifespan between female castes (i.e. workers and queens) with identical genome to gain insight into the processes of ageing.

Most previous studies using honey bees have addressed changes in workers during their transitions between different life-history stages (from in-hive nurses to foragers). Few studies have examined the large difference in lifespans between queens and workers to try to elucidate the mechanism/s of ageing. No studies have looked at the complete life-cycle history stages of honey bees that represent a unique opportunity to investigate ageing through their well-characterised development program (i.e. larva, pupa and adult stages), and there is virtually no information on the physiology of the honey bee male caste.

## 6.1 Major findings of this study

All castes were found to share a very similar membrane phospholipid composition during their development (larvae and pupae). However, post-emergence, membrane phospholipid fatty acid compositions differed significantly. These differences appear to be associated with changes in the diet of the different castes during their adult life. After emergence, worker bees begin feeding on honey and pollen whilst queen bees are fed royal jelly, mouth-to-mouth, by nurse bees for the duration of their adult life. The consumption of pollen by young adult worker bees resulted in a significant increase in the polyunsaturated fatty acid (PUFA) level of membranes. The increase of PUFA in worker membranes would increase their susceptibility to peroxidation (high membrane peroxidation index; Figure 2.1-D) compared to those of adult queens. The changes in membrane fatty acid composition

occurred relatively rapidly in worker bees (during the first four days post-emergence), with the incorporation of PUFA occurring in all classes of phospholipid molecules (see section 2.3.11). In contrast, the membrane phospholipids of adult queens remained highly monounsaturated throughout their adult life (up to 3 years measured) and subsequently were more resistant to oxidative damage (i.e. low membrane peroxidation index; Figure 2.1-D).

Examination of the phospholipidome of bees showed that although PUFA are universally spread across the different phospholipid head groups between castes, they are still differentially located among phospholipid headgroups (Figure 2.3). Adult workers had most of their PUFA attached to phosphatidylcholine (PC) and phosphatidylethanolamine (PE) molecules whilst adult queens had most of their PUFA attached under phosphatidylinositol (PIn), with a small contribution from PC and PE compared to workers (Figure 2.3). As a result of their lower PUFA levels, queens maintained a greater abundance of phospholipid molecules with saturated (SFA) and monounsaturated (MUFA) fatty acids, notably PC/ PE 18:1_18:1, PIn 18:0_18:1. These types of phospholipids have previously been shown not only to be resistant to peroxidation but also protective against peroxidation of PUFA-containing phospholipids (Cortie and Else, 2015). Therefore, compared to workers, queens had less peroxidisable membrane phospholipids surrounded by phospholipids with antioxidant-like action.

Adult queens possessed a higher abundance of their PUFA-containing phospholipids as plasmalogens, compared to the shorter living worker bees. Plasmalogens have been shown to limit lipid peroxidation (Engelmann, 2004) and may confer antioxidant protection to PUFA-containing phospholipids in the membranes of queen bees. The results contained within this thesis (plus that of previous studies; Haddad et al., 2007; Robinson and Nation, 1970) indicate that the increased longevity of queens compared to worker bees may be associated with peroxidation-resistant membranes. The present study also found that adult drones possess membrane phospholipid fatty acid compositions similar to those of adult queens, suggesting that given the opportunity, drones may live a longer life than workers (see section 6.4).

A detailed analysis of changes in membrane phospholipid/fatty acid composition for the first three weeks of a worker's adult life (Chapter 3) found that feeding on pollen (in

contrast to queens that are feed royal jelly only) increased the incorporation of PUFA in membranes. As previously mentioned, these changes occurred over the first four days of adult life, becoming relatively stable thereafter. The types of PUFA that increased in membranes were 18:2 followed by 18:3, the same two PUFA found in pollen (Table 3.1). The increased incorporation of PUFA came at the cost of reducing monounsaturated fatty acid levels, most notably 18:1 (Figure 3.4). The rapid incorporation of PUFA into membranes was also accompanied by a ~4-fold increase in the total level of phospholipids present in worker bees (Figure 3.2). This increase was initially dominated by *de novo* synthesis of phospholipid molecules that contained SFA and MUFA only. The PUFA-containing phospholipid molecules appeared to be slightly delayed in their synthesis but started to increase in their incorporation after 48 hours (Figure 3.1). The production of the PUFA-containing phospholipids came at the cost of reducing the newly synthesised SFA and MUFA containing phospholipids, with the net result being a stable but elevated level of phospholipids present in worker bees 48 hours post- emergence (Figure 3.2). Overall, the study showed that membrane remodeling in worker bees is fairly fast following emergence but then remained relatively stable. This membrane remodeling is likely to reflect increases in mitochondria and supporting membrane networks to allow flight endurance, and it also gives worker bees the ability to act as 'heater bees". Several insects (e.g. beetles, butterflies, bumble bees) warm-up their flight muscles before flight, but worker honey bees can use their flight muscles to thermoregulate their environment, regulating the temperature of the brood between 32-35°C (Tautz, 2008; Winston, 1987). The level of cytochrome *c* (i.e. the electron shuttle that transfers electrons from complex III to complex IV in the electron transport chain) increases progressively from emergence to 14 days in worker bees (Herold and Borei, 1963). Citrate synthase, a commonly used enzyme marker for the presence of mitochondria, has also been found to increase by up to 5-fold from emergence to day four (Harrison, 1986), suggesting workers increase mitochondria density after emergence. This increase in mitochondrial density appears to be maintained during adult life as mitochondria occupy approximately 43% of muscle fibre volume in forager bees (Suarez et al., 2000) and mitochondria volume also increases by 60% from pupa to 20-day-old adult worker bees (Herold, 1965).

As originally proposed in a previous study (Haddad et al., 2007), the longevity of queen bees compared to worker bees may be explained by nutritional differences during adulthood. The present study confirmed membrane phospholipid composition differences

between workers and queens but then went on to experimentally test this idea. The results of these experiments showed that the membrane phospholipids of worker bees are influenced by the lipid content of their diet in a predictable fashion (see Figures 4.1; 4.3 and 4.4). Accordingly, emergent workers fed a diet deprived of PUFA maintained a low membrane peroxidation index (similar to the level of adult queens) while worker bees fed a diet containing PUFA increased their membrane peroxidation index by 5-fold during the first week of their adult life. The difference in the workers' membrane peroxidation index was associated with differences in lifespan (Figures 4.7; 4.8; Table 4.4). Workers fed diets deprived of PUFA maintained a low membrane peroxidation index showed a trend towards an extension of maximum lifespan (average of longest living 10% of the population) for both diets tested (i.e. yeast and casein diets) compared to diets containing PUFA (i.e. pollen and casein+PUFA diets). The extension of lifespan was observed regardless of the protein ingredients used to make the diets (although not statistically significant for the yeast versus pollen comparison, $p = 0.07$). These results reinforce the possibility that dietary lipids may influence maximum lifespan by affecting membrane phospholipid composition and subsequently peroxidisability (Hulbert, 2005; Hulbert et al., 2014b, 2017). On the other hand, the average lifespan was similar in all diets, suggesting that the influence of dietary lipids on ageing is limited.

Although ageing relates to maximum lifespan, it is expected that a treatment that delays ageing will influence both average and maximum lifespans. An extension of maximum lifespan in worker honey bees, associated with differences in the membrane peroxidation index, is compatible with the predictions of the membrane pacemaker theory of ageing (Hulbert, 2005) where the membrane peroxidation index has been correlated with maximum lifespan in mammals, birds, molluscs, and some long-living strains of *Caenorhabditis elegans* (reviewed in Hulbert et al., 2017). Calorie restriction, which is the only experimental manipulation known to lead to a consistent extension in lifespan, is also associated with reductions in the membrane peroxidation index (Faulks et al., 2006).

Thus, the current results are among the first to demonstrate that a changing membrane peroxidation index could be associated with a difference in lifespan (see example of *Caenorhabditis elegans* below). The extension of maximum lifespan associated with a lowering in membrane peroxidation index suggests that workers would have some reduction

of oxidative damage (i.e. lipid peroxidation) compared to workers with a higher membrane peroxidation index. However, a reduction in the ability of membranes to propagate oxidative damage appears to have a significant influence only later on in life, as the average lifespan was similar in all dietary treatments. The regression between membrane peroxidation index and longevity of worker bees feeding on different diets is presented in Figure 6.1. The coefficient of the regression between lifespan and membrane peroxidation index is relatively low for average lifespan ($R^2 = 0.29$). However, the coefficient of the regression increases drastically for the longest 10%, longest 5% and longest-living individuals with $R^2 \approx 0.80$, supporting the notion that the membrane peroxidation index may have an influence later in life.

The increased lifespan observed in the longest living 10% of worker bees fed diets deprived of PUFA is unlikely to be explained by calorie restriction as worker bees had similar food consumption throughout the entire experiment. Metabolic rates and lipofuscins (used as biomarkers of ageing) were also similar between the treatments, suggesting that those parameters are also unlikely to explain the difference in lifespan between treatments. It is also important to acknowledge that the extension of maximum lifespan observed was derived from a single large experiment and further trials need to be conducted to confirm the findings. Although the extension in maximum lifespan was up to 30% greater for workers fed on the casein diet compared to workers fed on the natural diet (i.e. pollen), this extension is in the order of weeks, not the years as achieved by queens. Thus, it appears that membrane phospholipid composition is not the main causal mechanism for the shorter lifespan of worker bees. Overall, these experiments successfully tested the membrane pacemaker theory of ageing on worker bees, showing that the accumulation of polyunsaturated fatty acids in membrane phospholipids of worker bees may only partly explain their much shorter lifespan. These experiments do not rule out the possibility that the longevity of queens is still enhanced by the presence of peroxidation resistant membrane phospholipids. Another feeding study that examined the effect of dietary lipids on longevity of *Caenorhabditis elegans* showed reduced longevity, with 18:1, 18:2, and 18:3 additions resulting in average lifespans of 20, 8 and 14 days respectively compared to controls (no addition of fatty acids) at 22 days (Fang et al., 2016). Thus, while the addition of MUFA had a very small effect on longevity, the addition of PUFA resulted in a substantial shortening of lifespan. One way to partially test this hypothesis in honey bees would be to feed emergent adult queen bees a diet containing

PUFA, then observe its influence on membrane phospholipids and lifespan.

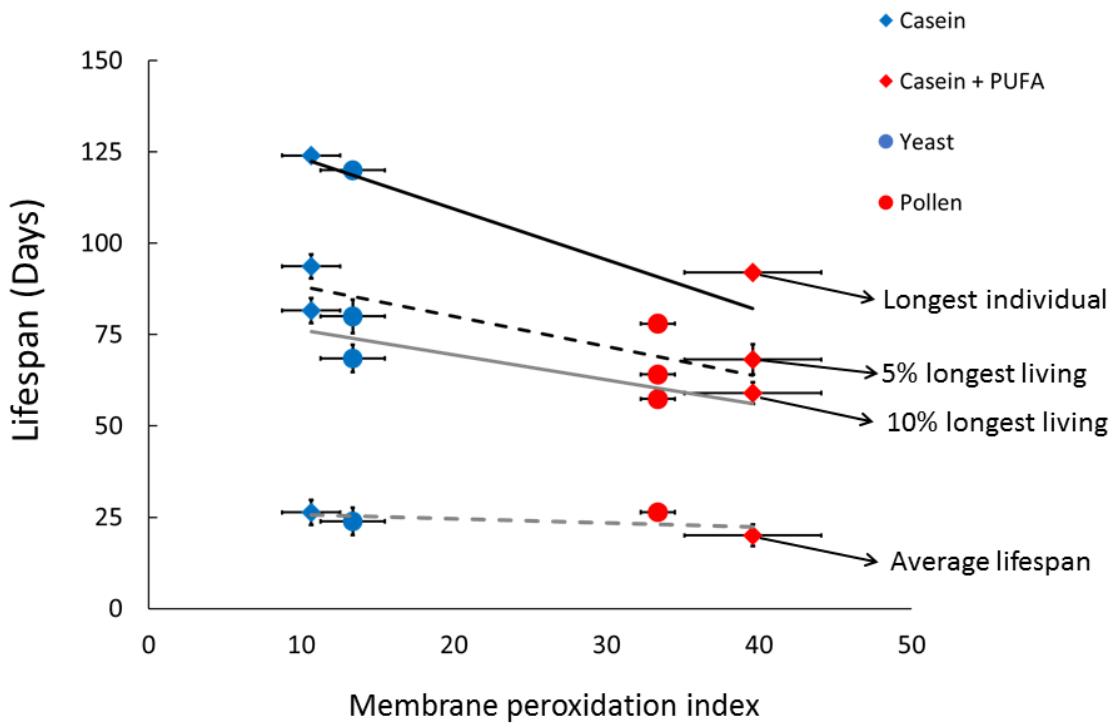


Figure 6. 1. Lifespan of worker bees (*Apis mellifera*) fed different diets relative to membrane peroxidation index (see Section 4.2.3 for diet details). Data are expressed as mean ± s.e.m. Lifespan is expressed in days. The four different diets are depicted in the legend. Average lifespan of whole population, 10% longest-living, 5% longest-living and longest-individuals are indicated for workers feed on Casein+PUFA diet as an example. The same scheme applies to other diets. The grey dash line represents the regression between average lifespan of the four groups and membrane peroxidation index ($R^2$ = 0.30). The solid grey line represents the regression between the longest 10% living-workers of the four groups and membrane peroxidation index ($R^2$ = 0.78). The black dash line represents the regression between the longest 5% living-workers of the four groups and membrane peroxidation index ($R^2$ = 0.80). The solid black line represents the regression between the longest living-worker bee for each group and membrane peroxidation index ($R^2$ = 0.81). See text for details.

The examination of lipofuscin fluorescent pigments (commonly used as a measure of cellular ageing in post-mitotic cells) in both female castes produced some surprising results. Firstly, during development, the level of lipofuscins was 8-fold higher in queens compared to

workers (Figures 5.1 and 5.4). However, these differences reversed during adult life with adult workers acquiring a significantly higher level of lipofuscins compared to adult queens. It was also observed that lipofuscins decreased during adult life in both female castes. This trend was observed in worker bees from a free-living colony (Figure 5.1) as well as worker bees maintained in dietary cages (Figure 5.3). The reduction in lipofuscins with ageing (which is contrary to most of the literature on this subject) suggests that both female castes have a mechanism(s) to dispose of this metabolic by-product. However, the difference in lipofuscins between the castes during adult life was fairly minor, suggesting that the burden of lipofuscins is unlikely to explain the difference in lifespan between workers and queens.

Female honey bees were also found to increase their routine metabolic rates with ageing. Just as membrane phospholipid compositions changed, the metabolic rate of worker bees also changed early in adult life. Worker bees increased their routine metabolic rate more than 4-fold during their first week of adult life. This elevated routine metabolic rate was subsequently maintained for at least the first four weeks of adult life (Figure 5.5). Queens also increased their routine metabolic rate during their first week post-emergence but to a lesser extent compared to workers (Figure 5.5). Older queens (one week and four weeks) had metabolic rates similar to those of age-matched workers. Another study has also shown that maximal metabolic rates (estimated during flight) are also similar between queens and workers (Harrison, 1986; Harrison et al., 2005). Thus, the "rate of living" hypothesis, which suggests that within animal groups those with higher metabolic rates are likely to have a reduction in lifespan, is unlikely to explain the longevity difference between queens and workers.

A common finding in the different physiological parameters measured in this thesis (membrane phospholipids, lipofuscins, metabolic rate) was for rapid changes to occur early-on in the adult life of worker bees. Interestingly, two enzymes used as markers for metabolism: pyruvate kinase (glycolysis) and citrate synthase (Kreb cycle/marker for mitochondria) increase by 10-fold and 5-fold, respectively, from emergence to four days (Harrison, 1986). In contrast, increases in metabolic rate (either maximal or routine) have previously been reported to be delayed reaching maximal values at 7-10 days (Chapter 5; Harrison, 1986), potentially influenced by the increasing number of flights in worker bees during their transition from nurses to foragers (Fahrenholz et al., 1992; Harrison, 1986).

These results indicate that typical comparisons of nurses versus foragers may miss some of the changes taking place early in adult life.

## 6.2 Mechanisms underlying the difference in longevity between workers and queens

The current feeding experiments were all performed on post-emergent worker bees. They were designed to change membrane phospholipid compositions of worker bees post adult emergence (see Chapter 2). However, the development of the larva into a queen, or a worker is determined before emergence and appears to depend on the amount of royal jelly available. Up to the third day, female larvae are bi-potent and can become either a worker or a queen (Haydak, 1970). Differentiation at larval stages involves epigenetic modification by DNA methyl transferases (Kucharski et al., 2008) associated with different epigenetic signatures (Dickman et al., 2013; Foret et al., 2012) and different transcriptomic profiles (Ashby et al., 2016; Foret et al., 2009) between the castes. Therefore, a potential mechanism(s) that influences longevity in honey bees could be determined before emergence. The influence of PUFA on lifespan at this developmental stage could be tested by supplementing royal jelly fed to larvae with PUFA.

## 6.3 Future research questions using the honey bee system

In honey bees, caste differentiation that occurs during the larval stage is associated with an excess of food in queens compared to workers. In other words, workers receive enough food to grow and survive, while queens are fed with excess royal jelly throughout their entire, but shorter, larval development. This difference in food allocation of up to 1,000-fold (Haydak, 1970) is thought to be the main factor that influences caste differentiation between workers and queens. Interestingly, this excess of food provides a phenotype that lives up to 100-fold longer. For decades, caloric restriction has been a key pillar of ageing biology, the fundamental tenet being that calorie restriction slows ageing. However, in the case of honey bees, an excess of royal jelly, which is highly nutrient-rich, leads to a phenotype that shows a dramatic increase in lifespan, countering to the tenets of calorie restriction. This observation begs the question about the mechanisms through which such “over nutrition” works, and suggests that the underlying mechanism(s) that explains the difference in longevity may be determined before adult emergence.

## 6.4 Long Live The Drone

The similarity in membrane phospholipid fatty acids between drones and queens during adulthood was surprising (Chapter 2). Drones are only produced during the warmest month of the year when virgin queens are available. There is little information available on drones generally (Robinson and Nation, 1970) although they are thought to feed on pollen during adulthood (Szolderits and Crailsheim, 1993b; Winston, 1987). However, the current results would suggest otherwise (Chapter 2) as the consumption of pollen would lead to an increase in membrane PUFA content, as found in workers. Drones have no specific functions except as "sperm donors" and are thought to have a short lifespan under free-living conditions (Rueppell et al., 2005), as worker bees usually expel drones from the hive after the mating season and reproduction is suicidal for drones, both of which shorten their lifespan. The lifespan of a drone might, therefore, be limited by the social behaviour of the hive, but if membrane composition is associated with longevity, then their membrane phospholipid composition would suggest that drones have the potential to live much longer than they do under free-living conditions. The maintenance of drones in captivity (as performed in Chapter 4) could be used to test this hypothesis, and drones may also represent an excellent alternative model to test the influence of membrane PUFA on longevity since drones have membranes similar to those of queens. Furthermore, those results strongly support that more data are badly needed for this neglected gender.

## 6.5 A final comment

This study has demonstrated that the extended longevity of queens compared to genetically identical workers is associated to some extent with peroxidation-resistant membranes as postulated that the difference in lifespan between the castes is related to a difference in nutrition during adult life. After emerging as adults, workers start feeding freely on pollen (high content of polyunsaturated fatty acid; PUFA) while the queens are fed royal jelly (negligible amount of PUFA) for life. Thus, if the extended longevity of the queen is associated with peroxidation-resistant membranes, this suggests that the feeding of royal jelly is an efficient way of keeping the queen away from the polyunsaturates in pollen, allowing her to maintain peroxidation-resistant membranes throughout her entire life. The difference in nutrition between the castes during adult life provides a unique opportunity to test the

influence of nutrition on a model that has evolved a tremendous difference in lifespan.

The membrane phospholipids of worker bees were found to be highly regulated by the PUFA content of diets. The difference in membrane phospholipids was associated with an extension of maximal lifespan (average of the longest 10% of the population) by up to 30% in workers that maintain low content PUFA in their membranes (just like adult queens) compared to workers that increase their membrane PUFA content. However, the lifespan of workers remained in weeks/months, not in years like as achieved by queens, suggesting that membrane fatty acid composition may only partially explain the difference in lifespan between the castes.

The ability to extend lifespan in worker bees by nutritional manipulation provides a valuable experimental tool to investigate the processes of aging. The research conducted as part of this *Ph.D.* indicates that models such as social insects deserve more consideration and offer a unique opportunity to understand mechanisms that have evolved to influence ageing without experimental manipulation.

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# Appendices

# Appendix 1 – List of precursor ion scans used for the lipidomics

| | Ion Mode | Scan | DP | EP | CE | CXP | Mass Range | Da/ second |
|---|---|---|---|---|---|---|---|---|
| *Phospholipid class scans* | | | | | | | | |
| PC | Positive | PI 184.1 | 100 | 10 | 40 | 8 | 640-850 | 200 |
| lyso-PC | Positive | PI 184.1 | 100 | 10 | 40 | 8 | 490-590 | 200 |
| PE | Positive | NL 141 | 100 | 10 | 30 | 8 | 685-950 | 200 |
| Lyso-PE | Positive | NL 141 | 100 | 10 | 30 | 8 | 420-540 | 200 |
| PS | Positive | NL 185 | 100 | 10 | 25 | 8 | 730-850 | 200 |
| PIn | Negative | PI 241 | 100 | 10 | 30 | 8 | 750-1040 | 200 |
| *Fatty acid chain scans* | | | | | | | | |
| 14:0 | Negative | PI 227.2 | 100 | 10 | 55 | 11 | 580-900 | 1000 |
| 16:1 | Negative | PI 253.2 | 100 | 10 | 55 | 11 | 600-900 | 1000 |
| 16:0 | Negative | PI 255.2 | 100 | 10 | 55 | 11 | 600-900 | 1000 |
| 17:0 | Negative | PI 269.3 | 100 | 10 | 55 | 11 | 560-900 | 1000 |
| 18:3 | Negative | PI 277.2 | 100 | 10 | 40 | 11 | 600-900 | 1000 |
| 18:2 | Negative | PI 279.2 | 100 | 10 | 40 | 11 | 600-900 | 1000 |
| 18:1 | Negative | PI 281.3 | 100 | 10 | 55 | 11 | 600-900 | 1000 |
| 18:0 | Negative | PI 283.3 | 100 | 10 | 55 | 11 | 600-900 | 1000 |
| 19:0 | Negative | PI 297.3 | 100 | 10 | 55 | 11 | 600-900 | 1000 |
| 20:5 | Negative | PI 301.2 | 100 | 10 | 40 | 11 | 500-1000 | 1000 |
| 20:4 | Negative | PI 303.2 | 100 | 10 | 40 | 11 | 600-1000 | 1000 |
| 20:3 | Negative | PI 305.2 | 100 | 10 | 40 | 11 | 600-1000 | 1000 |
| 20:2 | Negative | PI 307.2 | 100 | 10 | 40 | 11 | 600-1000 | 1000 |
| 20:1 | Negative | PI 309.2 | 100 | 10 | 55 | 11 | 600-1000 | 1000 |
| 20:0 | Negative | PI 311.2 | 100 | 10 | 55 | 11 | 600-1000 | 1000 |
| 22:6 | Negative | PI 327.2 | 100 | 10 | 40 | 11 | 700-1000 | 1000 |
| 22:5 | Negative | PI 329.2 | 100 | 10 | 40 | 11 | 700-1000 | 1000 |
| 22:4 | Negative | PI 331.2 | 100 | 10 | 40 | 11 | 700-1000 | 1000 |
| 22:3 | Negative | PI 333.3 | 100 | 10 | 40 | 11 | 600-1000 | 1000 |
| 22:2 | Negative | PI 335.2 | 100 | 10 | 40 | 11 | 700-1000 | 1000 |
| 22:1 | Negative | PI 337.3 | 100 | 10 | 55 | 11 | 700-1000 | 1000 |
| 22:0 | Negative | PI 339.3 | 100 | 10 | 55 | 11 | 600-1000 | 1000 |
| 24:1 | Negative | PI 365.3 | 100 | 10 | 55 | 11 | 700-1000 | 1000 |
| 24:0 | Negative | PI 367.3 | 100 | 10 | 55 | 11 | 700-1000 | 1000 |

Mass shifting was prevented in negative ion mode by increasing number of summed scans. *PI* precursor ion, *NL* neutral loss, *DP* declustering potential, *EP* entrance potential, *CE* collision energy, *CXP* collision cell exit potential, *PC* phosphatidylcholine; *PE* phosphatidylethanolamine; *PS* phosphatidylserine, PIn phosphatidylinositol, lyso lysophospholipid.

# Appendix 2. Composition of internal standard added to the lipid extracts.

| Molecular phospholipids | nmol per sample |
|---|---|
| LPC 17:0 | 15 |
| PC 19:0_19:0 | 80 |
| LPE 14:0 | 15 |
| PE 17:0_17:0 | 50 |
| PIn 18:0_18:0 | 10 |
| PS 17:0_17:0 | 15 |

LPC: Lysophosphatidylcholine; PC: phosphatidylcholine; LPE: lysophosphoethanolamine; PE: phosphatidylethanolamine; PIn: phosphatidylinositol; PS: phospholtidylserine. Concentrations are for post-emergent bee samples. For larvae and pupae, 50 % of the concentration values were used (e.g. 7.5 nmol for LPC 17:0).

# Appendix 3. List of all individual molecular phospholipids compiled in the three castes of honey bees (*Apis mellifera*) Data were quantified as nmol. mg $^{-1}$of bee.

| Headgroup | Fatty acids | |
|---|---|---|
| LPC | 16:0, 16:1, 18:0, 18:1 | 18:2, 18:3 |
| PC | 16:0_16:0, 16:0_16:1<br>16:0_18:1, 16:1_18:0<br>16:1_18:1, 18:0_18:0<br>18:0_18:1, 18:1_18:1<br>18:1_20:1 | 16:0_18:2, 16:0_18:3<br>16:1_18:2, 18:0_18:2<br>18:0_18:3, 18:1_18:2<br>18:1_18:3, 18:2_18:2<br>18:2_18:3 |
| LPE | 18:0, 18:1 | 18:2, 18:3 |
| PE | 16:0_18:1, 16:1_18:1<br>18:0_18:0, 18:0_18:1<br>18:1_18:1, 18:1_20:0,<br><br>O=16:0_18:1<br>O=16:1_18:1<br>O=18:0_18:0<br>O=18:1_18:1 | 16:0_18:2, 16:1_18:2<br>18:0_18:2, 18:0_18:3<br>18:1_18:2, 18:1_18:3<br>18:2_18:2, 18:2_18:3<br>18:3_18:3<br>O=16:0_18:2, O=16:0_18:3<br>O=16:1_18:2, O=16:1_18:3<br>O=18:0_18:3, O=18:1_18:2<br>O=18:1_18:3 |
| PS | 16:0_18:1, 16:1_18:0<br>16:1_18:1, 18:0_18:0<br>18:0_18:1, 18:1_18:1 | 18:0_18:2, 18:0_18:3<br>18:1_18:2, 18:1_18:3 |
| PIn | 16:0_16:1, 16:0_18:1<br>16:1_18:0, 18:0_18:1<br>18:1_18:1 | 16:0_18:3, 18:0_18:2<br>18:0_18:3, 18:1_18:2<br>18:1_18:3, 18:2_18:3<br>18:3_18:3 |

LPC: Lysophosphatidylcholine; PC: phosphatidylcholine; LPE: lysophosphoethanolamine; PE: phosphatidylethanolamine; PIn: phosphatidylinositol; PS: phospholtidylserine. O= indicates a phospholipid containing alkyl ethers (termed O=) or vinyl ethers (plasmalogens, termed P=) that could not be differentiated using the current method, see Section 2.2.3 for details.

# Appendix 4. Raw data for molecular phospholipids for Chapter 2 and Chapter 3.

Original raw data for the individual bee as well as a summary for all phospholipid molecules quantified is available upon request. Due to copyrights, the original content of the raw data generated in this thesis could not be included in this current document. Send an email to Dr. Nicolas Martin at mitochondriak@gmail.com, and I will happily assist you. Alternative contact is Professor Paul Else at pelse@uow.edu.au.